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A TECHNIQUE OF ORGAN CULTURE FOR PROTRACTED METABOLISM STUDIES

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THE DEVELOPMENT of a perfusion apparatus capable of sustaining life and growth in an excised organ has occasioned much study. Carrel and Lindbergh (1938) presented the technique as developed by them and their associates at that time. Most recently Long (1946) and Anderson and Long (1947) and Hetcher (1948) have shown the value of an in vitro study of organ function by this type of technique.

None of the above workers has been able to state that growth was grossly visible in their preparations. In their discussion Carrel and Lindbergh point out that the absence of red cells in the perfusion medium probably kept their preparations from exhibiting full function.

The work of Anderson and Long has been concerned chiefly with short time studies in which growth would be difficult to ascertain.

Our work has dealt with repeated studies on the same isolated organ perfused for several days. Each single study lasted approximately 24 hours. We felt that the data obtained would not be of real significance unless we could be certain that under the conditions imposed the organ was capable of showing growth or healing. We have directed the development of the technique toward that end.

Several specimens studied have demonstrated this capacity conclusively. As part of the study biopsies were made on the 2nd and 3rd

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[1] Obtainable from Dewey and Almy Chemical Company, Cambridge, Massachusetts.

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day of perfusion. Healing of the wound and closure of the biopsy aperture occurred 72 to 96 hours later. It seemed to us that a description of the technique in fairly extensive detail at this stage of development would be of value to other workers.

DESCRIPTION

To illustrate the apparatus a working diagram is presented in Figure 1. Figures 2 and 3 show the apparatus used for obtaining an alternating pressure and vacuum, and Figure 4 illustrates the type of cannula employed.

Since Figure 1 is not to scale the following dimensions of importance should be kept in mind.

1. The Erlenmeyer flasks from which the organ chamber and fluid reservoir are made are of 1000 c.c. capacity.

2. The pyrex tubing used is of heavy wall $\frac{1}{4}''$ internal diameter.

3. The rubber tubing has a bore of $\frac{1}{4}''$ with a wall thickness of $3/16''$. Only pure gum, autoclavable tubing is employed.

4. The rubber diaphragm (1) used in the pulsator tube (2) consists of the neck of a stratosphere balloon.[1] The function of the rubber diaphragm is solely to separate the perfusion fluid and the pulsating air. The apparatus could be operated without the use of the diaphragm but then a sterilizing bulb such as Lindbergh employed would have to be placed in the pulsating air column.

5. The valves do not have ground glass contact surfaces. They are highly polished to provide firm closure. The high polish reduces to a minimum the grinding and destruction of red cells.

6. The "rubber bushings" (3) used are of the "Fenwall" type. One closes the pulsator tube (2) and the other the organ chamber. Sufficient flexibility is provided by the latter to permit the arranging of the organ chamber in relation to the organ so that the organ can rest on the side of the chamber without undue stretching of the artery. The need for such flexibility is found essential in mounts with extremely small arteries. If torsion or jamming of the artery over the mouth of the cannula occurs, the rate of blood flow with the same activating pressure can be reduced as much as 80% of that possible with a clear passage.

7. The tapered joints used on the connector (14) are standard taper 10/30.

8. The organ chamber has been used in many shapes. The tube shown is quite generally useful for small organs. For large organs we use a chamber from which the large end opposite the cannula has been blown out and a ridged rim fashioned around it. An opening large enough to insert the hand can be provided when the flask is a 1000 c.c. Erlenmeyer. These large chambers are closed by fitting a surgeon's glove over the open end so that the wrist portion fits over the rim and the fingers project into the interior. The hand can then be

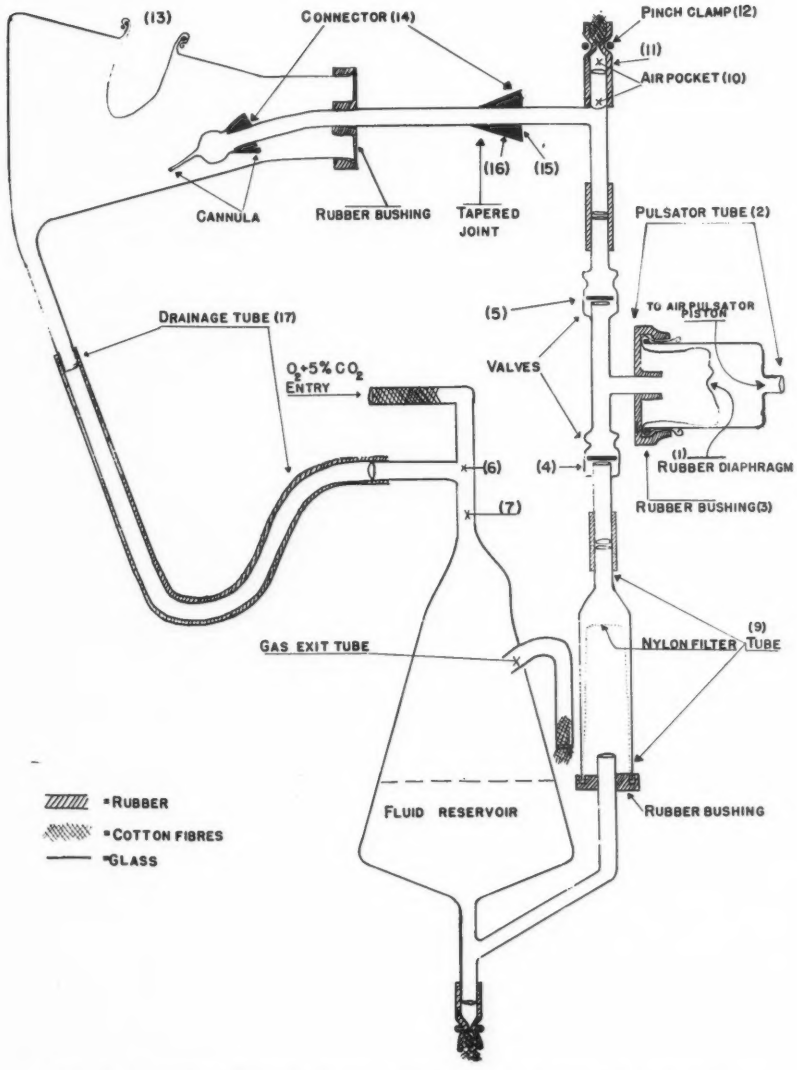

FIG. 1. Schematic diagram showing arrangement of parts of perfusion pump. Legend indicating nature of material is applicable throughout diagram except where so labeled in pulsator tube portion (2) and at the orifice (13) of the organ chamber. At these two points the single fine line indicates the use of a rubber condom or balloon neck as a flexible rubber diaphragm.

inserted into the glove and the organ and arteries palpated during a perfusion.

OPERATION

As can be seen from Figure 1 the two valves are so arranged that the fluids can circulate in only one direction. The lower valve (4) serves to prevent fluid from returning to the reservoir on the pressure stroke. The upper valve (5) which rises on the pressure stroke, holds the residual pressure during the pump's negative stroke and refilling of the rubber diaphragm. The fluid is forced into the artery through the cannula and emerges from the veins of the preparation.

As the organ chamber and blood reservoir are both at atmospheric pressure, the perfusate flows freely down the walls of the chamber and into the duct leading to the fluid reservoir.

Just prior to entry into the organ chamber the blood passes the mouth of the gas entry tube (6). The gas and blood flow down tube (7) into the organ chamber. Oxygenation and Co_2 removal from the fluid occur at this time. The process is usually complete by the time the fluid reaches the well of the fluid reservoir. The gas passes out of the chamber through the exit (8). A mixture of 5% CO_2 in Oxygen has been found requisite to maintain the pH at 7.2.

Since small bits of tissue, blood clots, etc. could cause a complete stoppage of flow, the blood is filtered in tube (9). The filter is a very fine mesh nylon bag.

The diaphragm chamber has a total volume of about 100 c.c. Of this volume about 50 c.c. is occupied by fluid within the diaphragm. The total volume of air being compressed is about 1000 c.c. Under the conditions of operation about 10 c.c. of fluid passes through the organ at each beat under maximum flow conditions and 0.02 at a minimum. Therefore, with a varying rate of blood flow the applied pressure on the organ can not change appreciably even though the change from minimum to maximum occurs within a few minutes.

The diastolic pressure is maintained by the air trapped at point (10). The volume of this air can be adjusted by introducing it with a sterile hypodermic needle through the rubber closure (11), or by allowing it to escape past the pinch cock (12).

The actual pressures are determined by a simple compression device inserted through the aperture (13). The device consists of a glass tube with a rubber diaphragm tied over one end. Fused to the tube is a glass rod which is bent in such fashion that expansion of the diaphragm causes it to push against the rod. The artery is placed between the diaphragm and the rod. The artery extending from the device to the organ is palpated by inserting fingers through the rubber glove previously mentioned as closing the end of the largest chambers. Pressure is exerted and measured by the usual sphygnomometer arrangement. The points of maintained turgor of the artery and the

onset of pulsation mark the diastolic and systolic pressure levels respectively. These observations, while accurate enough to demonstrate the tremendous pressure loss induced by the cannula orifice, are too inaccurate for satisfactory study of the flow problems involved.

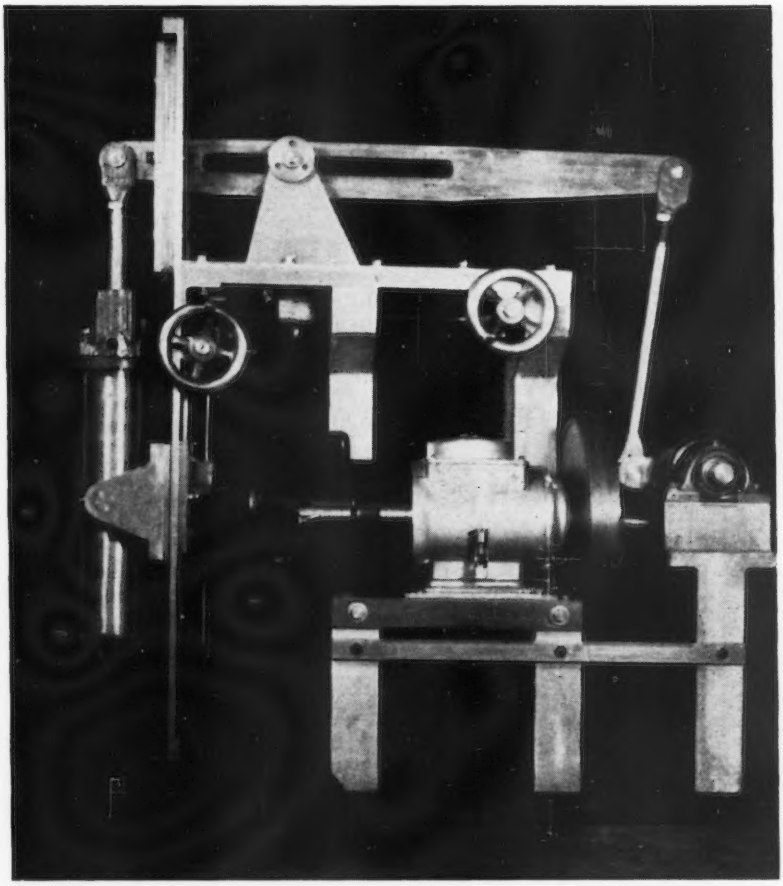

FIG. 2. Photograph of mechanical portion of actuating mechanism.

Efforts are being made to solve the problem of pressure measurement by electrical means.

BLOOD PRESSURE CONSIDERATIONS

In the work done previously blood pressure has often been measured in the glass portion of the apparatus. This type of determination was a feature of both the Long and Hechter and the Carrel and Lind-

bergh instruments. Under certain circumstances, such as when the arteries cannulated are large, this leads to but small error in the determination. When, however, the rate of flow thru the cannula is large so that friction loss is great, and when, as we have found preferable, the subsidiary arteries are not ligated, then a determination of the pressure anywhere but on the artery is relatively meaningless. It is obvious that under these circumstances that the actuating pressure must be very high to maintain 100 mm. of Hg pressure or more in the organ. It is for this reason that our apparatus has such a large capacity.

DRIVING MECHANISM

In Figures 3 and 4 are shown a photograph and diagram of the assembled apparatus illustrating the principles of operation.

This mechanism meets the following specifications:

1. It can deliver to the pulsator tube described earlier (No. 2 Figure 1). from 0 to 30 lbs. per sq. in. positive pressure and from 0 to 5 lbs. negative pressure on the opposite stroke of the piston.

2. Within requisite limits the positive and negative pressures can be adjusted independently of one another.

3. The rate of pulsation can be altered from 1 beat per minute to 150.

The amount of compression is regulated by moving the fulcrum point (1) Figure 3 along the slot (2) of the rocker arm (3). The amount of vacuum can be altered by either of two ways. First, by moving the cylinder (4) to a new position relative to the piston (5). This action increases or decreases the amount of "overshoot" of the piston in relation to its starting position. Secondly, air can be allowed to escape slowly from the system on the compression stroke. When the negative pressure on the return stroke equals that of the column of water in tube (7) air will again enter the system by bubbling up from the bottom of tube (8). The vacuum level can be further adjusted by regulating the height of the water column.

The apparatus was actuated by a $\frac{1}{2}$ horsepower motor driving the apparatus thru an adjustable gear ratio. The need for the massive construction is due to the fact that at 15 lbs. per sq. in. operating pressure the total thrust on the piston rod exceeds 400 lbs.

The handwheels shown in the photograph regulate the position of the walking beam fulcrum and the cylinder. Duplication of the settings for different experiments is facilitated by dials (not visible in photograph) mounted on the hub of the wheel.

We have found it requisite to use the water column arrangement continuously to maintain the setting of the apparatus. The most minute leaks will in time permit the loss of much air and so cause a drop in the actuating pressure and increment in the vacuum. The system as built works very effectively and its simplicity of construction and maintenance is a desirable feature.

In our earlier work, before developed the driving mechanism described above, we employed the method described by Carrel and Lindbergh and modified by Hechter. It was found to be adequate for the pressures they utilized but could not function adequately in the range which we found necessary for the reasons discussed above. When the

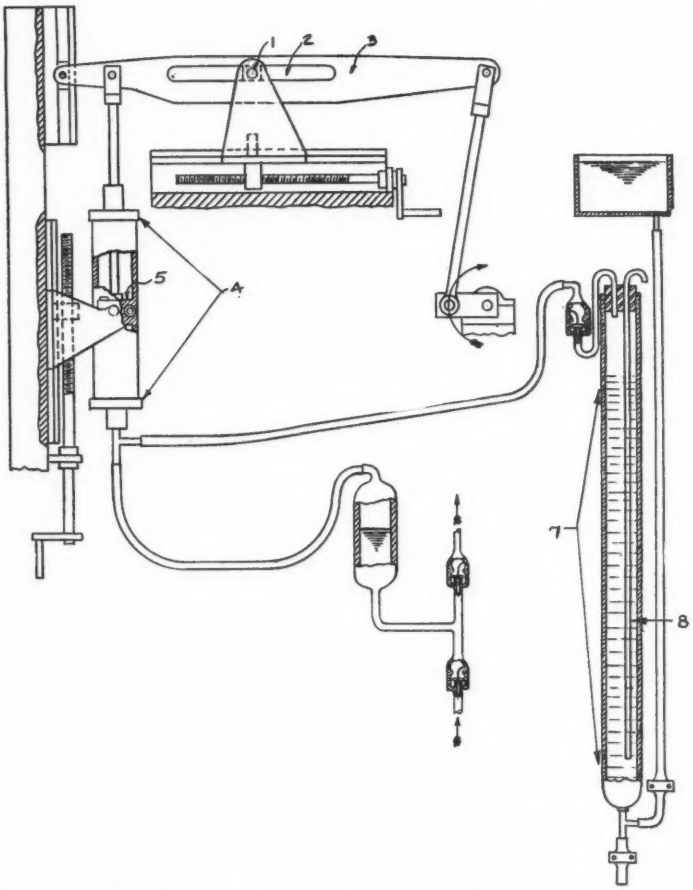

FIG. 3. Working diagram of actuating mechanism showing also relation to pump of "inverted" water column used to regulate vacuum and compressed air volume.

operating pressure in the fluid line had to be raised to 15 lbs. per sq. in. we found difficulty in obtaining surges in the oil vessel of an adequate intensity to give a sharp pulsation. Furthermore the amount of compressed air consumed at these operating pressures was enormous and called for very heavy duty compressors as a source of the operating pressure. While the initial expense of the apparatus described

is higher than that of the mechanical portion of the Carrel Lindbergh design or of Hechter's modification we have found that the greater reliability and ease of adjustment at the pressures we utilize makes the latter design mandatory for the long term perfusions.

STERILITY AND MOUNTING OF PREPARATIONS

Absolute asepsis, as pointed out by Carrel, is an essential of a successful prolonged perfusion. The elaborateness of the technique for prevention of infection must, of necessity, vary from laboratory to laboratory and is dependent on the conditions found in each institution.[2] We have found the use of glass shields between the operator and the specimen, a steam filled culture room, liberal application of chlorazene and alcohol to surfaces and instruments, adequate for success. Infections in the animals used as donors for blood and organs have proven more of a problem than technical failure.

As can be seen, the organ and culture fluid are not exposed to contamination once placed in the pump. If fluid is drained from the reservoir, the drain point can be sterilized again by washing out the tube with cotton soaked in a suitable germicide. Clamping of the tube and drying of the cotton prevent infection prior to the next draining.

New fluids and solutions of specific materials for study can be quantitatively introduced by injection through the thick walls of the rubber tubing employed. "Arterial-venous" differences in concentration may be ascertained by withdrawing samples of the perfusing fluid at the appropriate points in the cycle.

The operations involved in setting up the pump vary with the different organs and animals used as donors. In general we followed the technique described below:

1. Sodium sulphide is used as a depilatory. The skin is then washed thoroughly with hot water and 70% alcohol. Sterile towels are draped over those portions of the animal which have not been treated with the sulphide. The usual precautions for aseptic surgery are then followed.

2. After excision, the organ is placed in a large petri dish containing Tyrodes solution plus 10% by volume of a dextrose and sodium citrate solution.[3] The citrate solution is added to prevent clotting of the blood in the exposed blood vessels.

3. The cannula is inserted into the artery.

4. The artery is tied on to the cannula with number 00 surgical

[2] Carrel describes an elaborate procedure for asepsis. In our experience such elaborateness is requisite in dusty localities such as cities. Conversely in suburban or country regions few precautions are requisite. The work described here has been done in a suburb of Worcester. In the earlier stages of the development of this technique (1937 et seq.) while working in urban communities we too found an elaborate technique obligatory.

[3] Composition = 13.3 grams sodium citrate, 4.7 grams citric acid, and 30 grams dextrose, per liter.

silk thread. The loose ends of the tie are brought up over the bulge in the cannula which begins at the end of the grinding (Figure 4). At this point each end of the thread is tied to the cannula by a clove hitch. Tightening of this hitch forces the tie around the artery up the cannula until it is jammed against the increasing taper. Since the

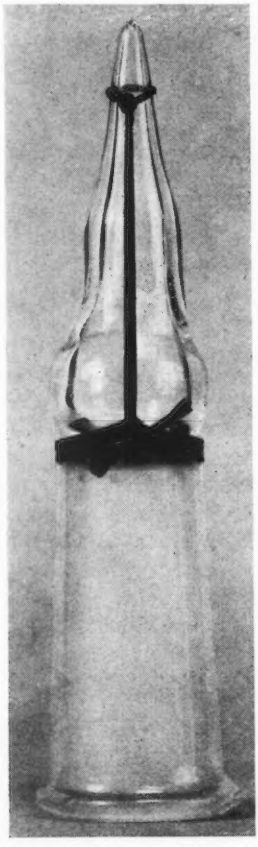

FIG. 4. Type of cannula inserted into artery of perfused organ. Manner of tying artery in position as described in text is also illustrated.

clove hitch knot will not stay tight permanently on a smooth surface the ends of the two threads are again passed around the cannula and knotted.

This combination of ties over (1) the tapered portion of the cannula and (2) the bulge makes certain that the artery will not slip off. The thread running from the bulge to the artery also serves as

an index of which side of the cannula should remain uppermost. While it is easy to see a twist in a large blood vessel, one can not always be certain of avoiding this difficulty when small vessels are employed.

5. After cannulation, the artery is flushed with dextrose citrate and Tyrodes solution to remove blood.

6. It is then attached to the connector marked (14) in Figure 1.

7. The organ is placed in the organ chamber through the mouth and the Fenwall stopper snapped into position.

8. The assembled unit is then carried to the incubator where the male joint (15) is inserted into the female (16) and the drain tube (17) connected to the blood return tube of the blood reservoir.

9. By alternately blowing and sucking on the tube leading to the compression chamber, the perfusion fluid is forced past the valves and up to the base of the air trapped at point (10).

10. The pump is then started on a pressure stroke.

We do not find that air emboli constitute a problem.

Only cannulated vessels of the size of the rabbits' femoral artery need to be tied. All the subsidiary arteries can be left open. Naturally such "leaks" increase the rate of blood flow so that settling out of the red cells does not occur. They also make easy the removal of air from the specimen if any is trapped. (In practise they also serve another purpose, that of estimation of the blood pressure in the perfusion of organs whose arteries are too small to permit palpation.). Obviously "hemorrhage" from an artery should squirt out if the blood pressure were normal. The height of the squirt is a function of the pressure. The fact that squirting occurs does not, of course, reduce the blood pressure to those branches of the cannulated artery which lead to the tissue being perfused. Connective tissue frequently overgrows these open ends, but a touch usually dislodges the overgrowth.

PERFUSING FLUIDS

It was found necessary to use red cells to obtain growth. Between 0.5 and 2 million per c.c. appear adequate. Blood is now drawn from the donor animal into a Fenwal flask and kept from clotting by using the citrate solution in a ratio of 1 c.c. citrate to 5 c.c. blood. The mixture is then diluted by half with White's solution and if clotting does not occur, calcium chloride crystals are added until clotting takes place.

The clots which form are broken up by shaking. Filtration of the blood through the standard nylon bag arrangement removes the clot fragments. The needle at the end of the filtering set-up (see below) is inserted into the drain tube from the organ chamber. This arrangement permits the filtering of the perfusing fluid and filling of the fluid reservoir at the same time.

At first we tried to use whole blood after clots had been removed.

All too frequently after several hours (10–12) of perfusion we found the blood beginning to clot again.

Our attention being at one point turned from animal organs to human organs, we were led to the technique finally devised. The blood for these perfusions could most expeditiously be obtained from a blood bank. Such blood contained 25% by volume of the citrate and dextrose solution mentioned previously. After inducing clot formation by adding $CaCl_2$ to 500 c.c. of citrated human blood, 250 c.c. could be drawn off. To increase our supply it seemed expedient to dilute the blood and Tyrodes solution was first employed. We did not find growth in perfusions set up with it as a diluent.

White's solution[4] (1946) was next used as diluent. It was found immediately that more $CaCl_2$ had to be added to such a mixture to induce clot formation than when Tyrodes had been used as diluent. However, once the mixture had clotted no further clots formed during a perfusion. It was with this mixture that growth was obtained. Six hundred to 700 c.c. of perfusion fluid containing 1 to 2 million red cells per c.c. became available after filtering off the clot when 400 c.c. of blood was diluted by 500 c.c. of White's solution and 100 c.c. of the dextrose citrate solution.

Obviously a solution providing less than 50% of the normal colloid osmotic pressure and only 1 million red cells per c.c. could stand improvement. Further work should be done to determine the amount of protein requisite to obtain maximum response. Good methods for the liberation of red cells from the clot are also needed.

HANDLING OF THE SOLUTIONS

The volume of sterile fluid employed in this technique is far larger than is generally used in tissue culture techniques. To expedite the process certain methods worthy of mention have been worked out.

We have found that the blood bank equipment designed for the "Fenwal System" is extremely convenient. This system centers around the use of a flask with a large mouth. Thru the use of a stopper such as we employ in the pump described earlier, various closures of the sterilized flask are available. One of these permits introduction and removal of fluid by hypodermic needles. We have found this closure extremely helpful. Use of a double needle permits filtration under vacuum of White's solution when large quantities (500 c.c.) are to be filtered. For smaller volumes, the vacuum present in the flask after autoclaving suffices. Only pyrex utrafilters are used.

Since we do not use Tyrodes solution alone in our work and always in an atmosphere of 5% CO_2 we employ a simplified method of sterili-

[4] White's solution is a completely synthetic medium capable of maintaining growth of various tissues for 1 to 2 weeks. It contains all the essential amino acids, glucose and vitamins found requisite by White in his remarkable study. We employ it as described by him except that we do not add the phenol red he employs as a pH indicator.

zation which involves autoclaving of the bicarbonate fraction. Such autoclaving is not ordinary practise as loss of CO_2 and an increment in pH results.

The solution was made up in 3 parts—A, B, and C as listed below.[5] Two test tubes of 20 c.c. volume were placed in the Fenwal flask. Into these were pippetted 10 c.c. each of solutions B and C. The largest volume (180 c.c.) containing 10 c.c. of A and 170 c.c. H_2O is placed in the body of the flask.

By keeping the flasks vertical during autoclaving and storage the 3 solutions are prevented from mixing. Tilting the flask several times before use mixes the solutions and a "fresh" solution of Tyrodes is available. This method of preparation permits the manufacture and storage of the solution in bulk quantities for prolonged periods. This is a tremendous advantage as occasionally the changing and washing of a pump for a new experiment on an organ involves the use of as much as 1 to 1.5 liters of Tyrodes solution.

Transfer of fluids from containers into the pump is made by the use of the drip technique employed in blood transfusions. When the mixture of White's and blood was transferred to the pump a nylon filter similar to the one employed in the pump is inserted into the line. Such prefiltering reduces the load on the filter in the pump and increases the time during which the filter can function.

DAILY ROUTINE OF ORGAN MAINTENANCE

The fresh perfusion fluid is removed from storage and placed in the incubator to reach the proper temperature.

A sample of the perfusing fluid is removed from the pump by hypodermic syringe and examined for bacteria.

If bacteria are seen or suspected 20 milligrams of streptomycin and 100,000 units of penicillin are added to the perfusion fluid. Experience indicates that in many instances this action stops bacterial growth.

If no bacteria are found the pump is drained, Tyrodes run in and pumped thru the organ. Several flushings are made in this fashion and the preparation is ready for another experiment. The changeover takes about 15 minutes and it is not requisite to interrupt operation during that period, altho cessation for a short period permits more accurate withdrawal of the perfusate.

If bacteria are found present the organ is installed in a new pump after the surface of the organ has been washed thoroughly with Tyrodes solution containing streptomycin and penicillin. After reinstala-

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[5]

Solution A 10 c.c.			*Solution B 10 c.c.*
NaCl	160.0 grams per liter	$NaHCO^3$	20.0 rams per liter
KCl	4.0 grams per liter		
$CaCl_2$	4.0 grams per liter		
$MgCl_2.6H_2O$	2.0 grams per liter	*Solution C 10 c.c.*	
$NaH_2PO_4H_2O$	1.0 grams per liter	Glucose	20.0 grams per liter

tion the antibiotics are added to the perfusate and the operation continued for 24 hours.

We find that in order to maintain the organs in good condition the perfusing fluid ought to be changed every 24 hours. At least 200 c.c. of medium is requisite to last thru this period.

To those acquainted with Carrel's work it may appear wasteful to change the fluid in the pump every 24 hours. His data indicated that the perfusion medium could be used for several days with the pH dropping as low as 6.9. We have not yet had the opportunity to study this in detail, as we were more interested in metabolic studies, than in organ survival under adverse conditions. A 24 hour period of perfusion was convenient for our work.

No studies have as yet been made on the rate of removal of the amino acids etc. present in White's solution. Such work has for the present been outside our province.

The gross changes visible in the circulating medium are, however, sufficiently marked to indicate the need for frequent alteration. A scum similar in appearance to that seen on infected serum frequently shows up after 24 hours of perfusion in perfectly sterile preparations. At the end of 72 hours of perfusion it is usually found necessary to transfer the organ to another pump because of filter blockage and inability to wash out the "scum" stuck to the walls of the fluid reservoir.

In several instances it has been possible to see gross changes in the organ when the fluid has been changed, brighter color, better blood flow, etc. One remarkable response has been an extreme contraction of the psoas muscle without other stimulation on the 4th day of a perfusion.

Therefore, while we have no concrete evidence at the moment as to the need of frequent change of perfusate, it is our sentiment that such work will provide data indicating the need for addition of food material and removal of waste products at frequent intervals.

OBSERVATIONS ON ORGAN VIABILITY AND NORMALITY OF FUNCTION

As mentioned earlier, our primary interest in the development of the technique described above has been the furtherance of the study of the metabolism of the steroid hormones. These studies have received our primary attention and we have neglected completely to make any studies of the protein, glucose, amino acid or vitamin metabolism under the imposed conditions.

We have had the problem, however, of validating our findings on the steroid hormone metabolism in the sense that experiments done on dead or dying tissue would be of small value in comprehending the normal situation. It appeared to us that growth of the organs, particularly in the cut portions, would be an adequate criterion of success in life maintenance.

Such growth is difficult if not impossible to describe for publication unless periodic photographs of the organ are taken during the experiment. It will not be further elaborated upon here except to point out that in several instances where the perfusion has lasted for sufficient time, almost complete healing of cut surfaces has occurred. In one perfusion of a human skin rope graft the connective tissue growth in 48 hours was such that at least two cubic centimeters of new tissue was formed around the cannulated blood vessel. Since no bleeding occurred at that point nourishment of the growing tissue could only only have come from a functioning vascular bed.

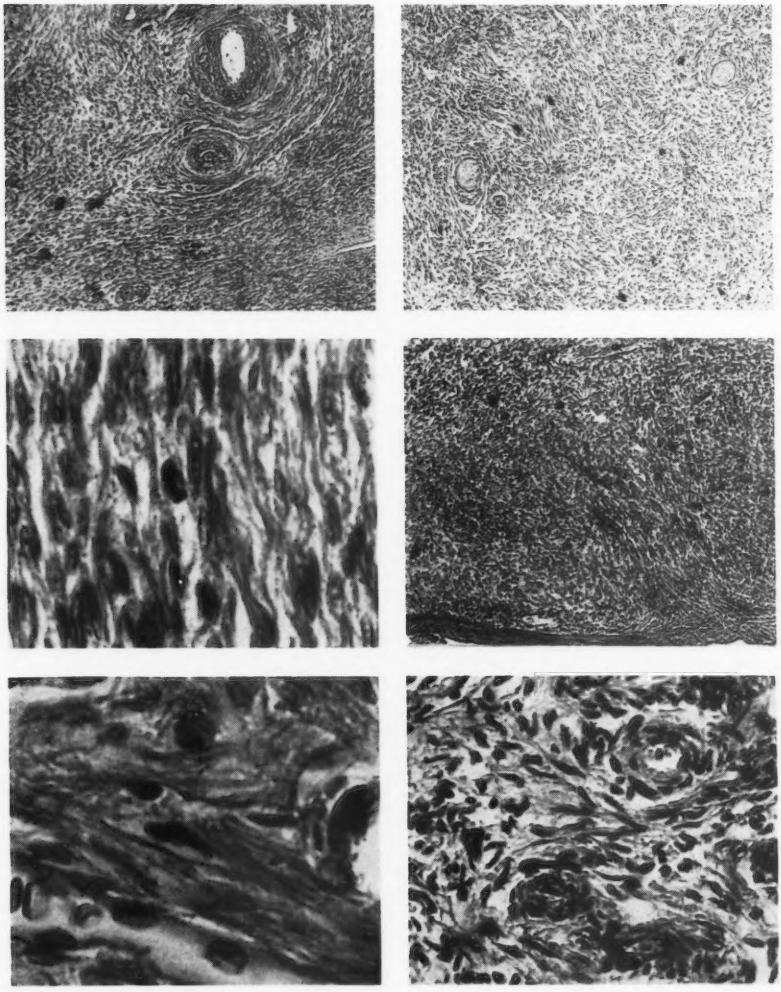
We have adopted the practise more recently when working with human tissue to remove a portion for section prior to the perfusion and comparing it with a specimen obtained at the end of the run. This technique is only of value when the experiment is terminated without an infection having entered the system. Very short exposures to an infection spoil the experimental specimen.

Figures 5 and 6, show the histological appearance of a human ovary and human muscle before and after perfusion. The muscle was fixed after a heavy infection had occurred. Twenty-four hours

FIGURE 5

Control, Human Ovary. Four sections representing ovary, tube and fibro-fatty tissue. The ovary reveals one moderately sized Graffian follicle containing a small amount of blood and detritus in the lumen. The lining is broken in several places but is made up of several layers of large cells arranged in moderately compact fashion. The cells themselves have ovoid to circular nuclei containing a moderate amount of nucleoplasm. The cytoplasm is coarsely reticular and in places granular. This represents the theca interna. The theca externa shows a layer of fusiform cells resembling fibroblasts and having elongated fusoid nuclei devoid of definite nucleoplasm. They give the appearance of being compressed because of their form and position. Fibrillae are present but not abundant. There is a large network of capillaries in this region. The remaining ovarian tissue shows a fair amount of stroma made up of the normal fusiform cells and fibrous connective tissue. The blood vessels are abundant and thick walled. The section of tube shows a mucosa which has lost its normal pattern. The interlacing branching formations are considerably reduced. Here the formations are thickened with fibrous connective tissue surrounded by low columnar and in places ciliated epithelium. No stratification is seen. There are areas which appear to be sneered off by the fibrous connective tissue preservation and this has greatly reduced the size of the lumen. These sneered off areas have large irregular acini-like spaces with broken thin partitions dividing them. The remaining tube wall is not remarkable, except for the great number of small thick-walled blood vessels and fibro-fatty tissue indicative of old chronic inflammation. Exudative cells are absent.

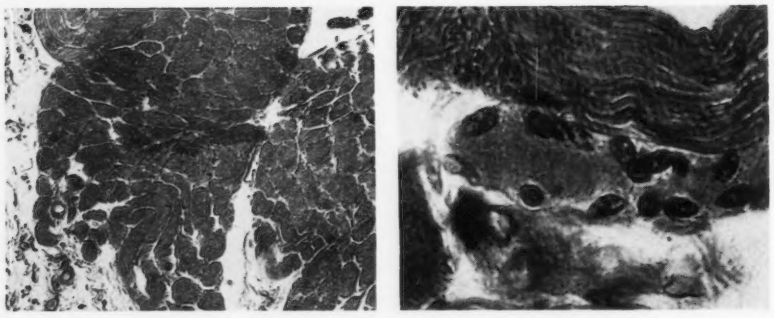
Experimental, Human Ovary. These sections of ovary show for the most part fairly good preservation of normal cellular architecture. There is moderate edema throughout. One involuting luteal cyst present. This has a peculiar amorphous architecture with columnar and pseudo-columns of large lipoid-laden cells either devoid of nuclei or having small deeply staining irregular nuclei. The cytoplasm when present is for the most part reticular or granular. In some of these areas center cores of fibrous connective tissue can be seen. The surrounding tissue is fibrous in character. The remaining ovarian tissue shows irregular, for the most part elongated, fusiform and in places wavy nuclei. There are many fibrillae but the edema renders the stroma less dense than normal and everywhere one encounters small nuclei suggestive of degeneration and necrosis. The blood vessels are thick-walled and fairly well preserved. Some contain agglutinated and conglutinated blood. Thrombosis is not a prominent feature.



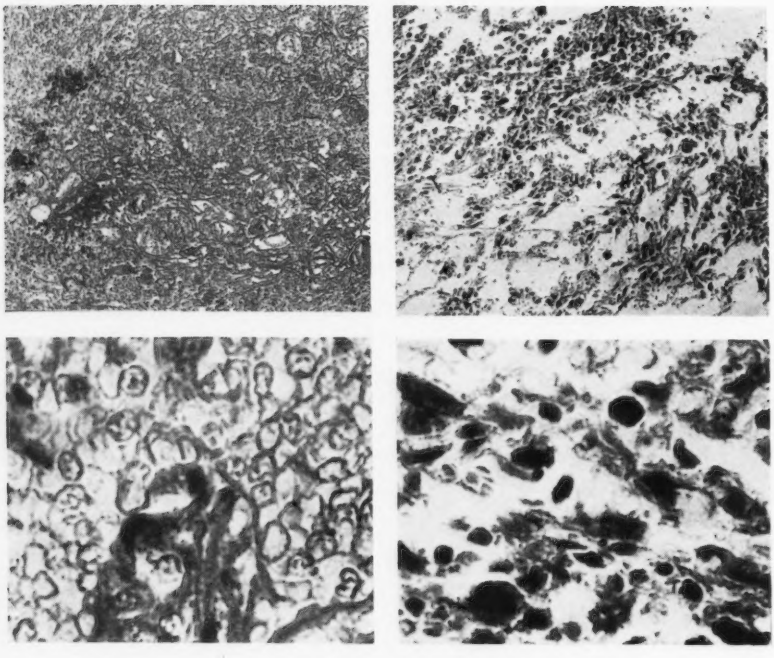
CONTROL TISSUE HUMAN OVARY EXPERIMENTAL TISSUE

FIG. 5. Infection found 6 hours before taking specimen. Perfused 120 hours. During 3rd. day of operation cannula and organ were blocked by clot formation. This was released by forcing Dextrose Citric Acid solution thru organ. Sheep Pituitary Gonadotrophin (500 R.U.) was added to perfusate on 4th day, (see table). Several areas of ovary showed infarcts on gross examination indicating incomplete perfusion. (Pathologist's description of tissue is shown on the facing page.)

previous to taking the specimen our notations on the latter experiment indicate a continuing growth of connective tissue. Appended to each of the photomicrographs is a description of the tissue as pre-



Control Tissue



Perfused Tissue
Human Muscle

Fig. 6. Human muscle taken from near tumor site. Heavy infection found in perfusate 12 hours prior to biopsy. Perfused 96 hours. (Pathologist's description is shown on next page.)

pared by Dr. Kaneb. These descriptions tend to indicate a decrement in the viability and general health of the specimen after several days perfusion even in the ovary. It must be recognised, however, that in each instance the perfusion was halted because of the presence of

bacteria in the perfusate. Consequently toxic materials were present. Considering, however, that toxins, "anemia," and inadequate protein concentration in the perfusing fluid all tend to reduce the probability of viability, it is indeed surprising that the specimens can look as well as the ovary shown.

Physiological evidence of function is available in the response of ovaries to gonadotrophin. We have been able to induce ovulation in cow and rabbit ovaries by the addition of large amounts of gonadotrophin to the perfusate. This has not been observed in human ovaries but here an increment in the estrone concentration of the perfusate has been seen as shown in Table I.

TABLE I

Ovary	Extraction	Days of Perfusion	Gonadotrophin Added	Total Estrone[6] Concentration in perfusate in micrograms
Ovary[7]	31	1	0	16
	32	2	0	16
	33	3	0	21
	34	4	500 R. U.	55
	50	5	0	26
Ovary	51	4	500 R. U.	36
	53	5	0	26

These several observations, histological, grossly visible growth and healing, functions such as ovulation and response to hormonal stimulus lead us to assume that our preparations provide a sufficiently normal milieu to make the data obtained applicable without too many reservations to understanding of the normal processes.

[6] Extracted and assayed as reported by Werthessen, Baker and Borci in 1947.
[7] Sections of this ovary shown in Figure 5.

FIGURE 6

Control, Human Muscle. These sections show for the most part skeletal muscle, fibro-fatty supporting stroma, nerve tissue and blood vessels. The skeletal muscle shows appreciable edema with conspicuous outlining of the individual muscle fibers and sarcolemal surrounding sheath. The individual fibers have in the longitudinal axis the normal cross striations. The nuclei are peripherally placed, oval and only fairly well preserved. In cross section there is appreciable fragmentation of the fibers. The stroma likewise shows edema and is made up mainly of loose fibrous connective tissue. Fat is not remarkable. Nerve elements are not unusual. Blood vessels are for the most part thickwalled and have a narrow lumen. Exudative cells are not present. In an occasional field appreciable degeneration is seen so that the cells appear to have lost their normal topography and become fragmented and almost homogeneous in appearance. No tumor is identified.

Experimental, Human Muscle. These sections show almost complete necrosis of the tissue elements. The transformation is such that the muscle elements are replaced by agglutinated and conglutinated blood through which irregular fibrillar streaks are seen and an occasional fibroblast is identified. Small blood vessels resembling capillaries lie here and there. To one side of these sections one area showing loose fibrous connective tissue with fusiform cells and a few round cells can be made out. The last mentioned tissue appears viable but resembles an inflammatory reaction and not neoplasia.

While tissue culture techniques have achieved growth of cells and in many instances remarkable developments of structure the cited experiments are the first in our knowledge wherein growth, repair, and function of whole adult organs has been at least partially maintained over a period longer than 24 hours.

There is, therefore, now available the elements of a technique which on further development will permit the study of isolated organ systems in a close to normal milieu.

SUMMARY

A technique for the maintenance of whole organs in a perfusion pump has been described. At its present stage of development growth of the organ and repair of injuries due to biopsies have been noted.

The extent of time during which the organ can be maintained is dependent only on the development of an uncontrollable infection.

The perfusion medium contained erythrocytes. The pump employed had a relatively large capacity permitting the circulation of the fluid at proper arterial pressure without ligation of arterioles. The high flow rate resulting prevented sedimentation of the red cells.

To the use of erythrocytes in the perfusion medium, the high flow rate and maintenance of normal arterial pressure were ascribed the ability to observe growth in the preparations.

The pump as finally developed was of simple construction and permitted easy access to the perfusion fluid and organ during the course of an experiment.

ACKNOWLEDGMENTS

I wish to thank Mr. Myles Morgan of the Morgan Construction Company for his invention of the driving mechanism employed in this technique.

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THE RELATIONSHIP OF TEMPERATURE AND INSULIN DOSAGE TO THE RISE IN PLASMA AMINO NITROGEN IN THE EVISCERATED RAT[1]

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THAT INSULIN will depress the rate of accumulation of amino acids in the blood of eviscerated rats was reported by Frame and Russell (1946) and confirmed by Ingle, Perstrud, and Nezamis (1947). However, the results of the latter authors differed from those of Frame and Russell in two respects: The rate of increase of the blood amino nitrogen content observed by Ingle et al. was about one third of that reported by Frame and Russell, and the rise was suppressed completely by much smaller doses of insulin than those which Frame and Russell found to suppress it only partially. Ingle et al. demonstrated that these differences were not the result of their having employed complete rather than functional evisceration, nor of the use of fed instead of fasted rats, nor of hemodilution. They suggested that possibly the differences could be accounted for by differences in methods of chemical analysis, for they had used for the estimation of amino nitrogen the ninhydrin-carbon dioxide method of Hamilton and Van-Slyke, whereas Frame and Russell had employed the photometric β naphthoquinone method, which is less specific. Another difference in the techniques used by two groups of investigators was the temperature at which the eviscerated rats were kept during the period of observation. Ingle et al, kept their animals in a chamber maintained at 26°C; under these conditions, the body temperatures of eviscerated rats fall in a short time to within a degree or two of that of the ambient air. Frame and Russell maintained the body temperatures of their animals at about 38°C.

It has now been found that the differences in the results of the two groups of experiments may be accounted for in large part, or perhaps completely, by the differences in body temperatures at which the observations were made. A small increase in the body temperature of the eviscerated rat will not only increase the glucose tolerance, as

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[1] The work reported in this paper was done under a grant from the American Cancer Society on the recommendation of the Committee on Growth, National Research Council.

Ingle noted, but will increase the rate of release of amino acids into the blood. The absolute depression of the rise in plasma amino nitrogen by a given dose of insulin at 27° and 38° is of the same order of magnitude, but since the increases without insulin are so much less at the lower temperatures, the relative response to insulin here appears to be greater than at the normal body temperatures.

METHODS

Adult male white rats 3 to 4 months old and weighing about 350 gm. were fasted 18 hours and then functionally eviscerated under pentobarbital anesthesia. In this operation the intestinal tract, spleen, and pancreas are removed, and the liver, left *in situ*, is effectively excluded from the circulation. The operation, which takes only a few minutes, can be performed without preliminary ligation of the vena cava and without any hemorrhage either at the time of operation or later. The initial dose of sodium pentobarbital, 4 mg. per 100 gm. given intrapertioneally 10 to 20 minutes before operation, sufficed for continued light anesthesia throughout the experiment. Rectal temperatures, deep within the pelvis, were measured at frequent intervals. They were maintained at 38 $\pm$ 0.5°C by means of lights placed 30 to 40 cm. above the animals. In the series in which lower temperatures were desired, the animals were exposed at a room temperature of about 24°C; here the body temperatures fell slowly during the interval between 1 and 4 hours after evisceration from about 30° to about 28°C. The rectal temperatures were found at the end of the experiments to coincide with those elsewhere in the body.

Glucose and insulin were given to the animals subcutaneously at hourly intervals beginning 15 to 30 minutes after evisceration. Glucose solution, 4 to 18 per cent in physiological saline, was injected in two portions totaling in volume 0.5 ml. per 100 gm. per hour, the insulin (Lilly amorphous or zinc crystalline) also in saline, in a volume of 0.1 ml. per 100 gm. per hour at a separate injection site. The amount of glucose given was adjusted to the amount of insulin so that the blood sugar level was maintained within the normal range. Animals not given insulin or given not more than 0.05 units per kg. per hour received 20 mg. glucose per 100 gm. per hour; those given 0.1 unit insulin per kg. per hour required 25 to 30 mg. glucose per 100 gm. per hour, 0.25 units, 40 mg. glucose, and 1 unit insulin, 80 to 85 mg. glucose. Blood glucose determinations were made at intervals during and at the end of the periods of observation. Analyses were made on 0.1 ml. of blood by the Nelson-Somogyi method. With the regimens described above, the blood sugar concentrations were in most cases between 90 and 140 mg. per cent and appeared to remain fairly uniform throughout each experiment.

Estimations of amino nitrogen were made on plasma rather than on whole blood, inasmuch as the high content of amino nitrogen in the red cells appears not to change much except in terminal anoxia. Analyses were made on 0.2 ml. samples of oxalated plasma by the photometric procedure of Frame Russell, and Wilhelmi (1943), as modified by Russell (1944).[2] In these experi-

[2] For the preparation of the tungstic acid filtrates from plasma, the following procedure was used: 0.2 ml. plasma was mixed in 5.0 ml. of 0.01 N sulfuric acid, then 0.2 ml. of 3.0 per cent sodium tungstate was added and mixed immediately. After the mix-

ments, observations were made at 1 hour and again at 4 hours after evisceration, and the increases in plasma amino nitrogen occurring during the three hour interval compared in the several series of experiments.

Blood samples were taken from the tail except at the end of the experiment, when they were usually drawn from the inferior vena cava. All animals were carefully observed during the experiment and at its end, and any showing any signs of anoxia or hemorrhage, or in which the body temperatures were out of the desired range, were discarded.

Ingle et al. reported much longer survival after evisceration when the rats were kept at lower temperatures, this being the reason for their having carried out their experiments under these conditions. No attempt was made in the present experiments to determine the survival times of the animals. A distinct difference in the conditions of the animals at the higher and lower temperatures was observed, however. The animals which were kept warm remained in excellent condition throughout the experiments. Those maintained at room temperature frequently shivered, exhibited peripheral vasoconstriction so that they could not be made to bleed from the cut tail, and in many cases may have been in some degree of shock. Despite the longer survival of the cold animals, it may be doubted that their state was as nearly normal as that of the rats which were kept warm.

RESULTS AND DISCUSSION

A comparison of the rate of rise of the plasma amino nitrogen after evisceration at 38°C with that at 29°C is shown in Table 1, where it may be seen that the increase at the higher body temperature is greatly in excess of that at the lower. The temperature coefficient would be for the conventional Q_{10}, 2.6 fold; or more exactly, if the slope of the line relating the natural logarithm of the plasma amino nitrogen rise to the temperature is calculated, 9.1 ± 0.7 per cent per degree centrigrade (see Figure 2). This increase in rate with temperature is of the same order of magnitude as that commonly observed for a wide variety of chemical reactions and physiological processes and would be an expected value for the continuing net enzymatic breakdown of tissue proteins.

The increase in plasma amino nitrogen in the eviscerated rat at 38°C reported here, about 14 mg. per cent in 3 hours, agrees well with that observed previously by Frame and Russell, about 18 mg. per cent in 4 hours. Ingle, et al. found an average increase of whole blood amino nitrogen of 5.5 mg. per cent in the first 6 hours after operation and 18.5 mgm. per cent in 24 hours, at body temperatures near 27°C. These figures would correspond to increases in plasma amino nitrogen of about 4.6 and 3.9 mgm. per cent in 3 hours, over the two time intervals respectively, if the average hematocrit were taken to be 40

ture had been let stand until settling had begun, the precipitated proteins were centrifuged off, and duplicate 2 ml. portions of the supernatant were used for analysis. With the naphthoquinone amino nitrogen method, to obtain quantitative recovery of added amino acids it is essential to use less tungstate for plasma than is ordinarily used for whole blood.

per cent, and if no increase occurred in cell amino nitrogen. These figures are to be compared with a mean increase of 5.9 mgm. per cent (standard deviation 1.1 mgm. per cent) obtained in 3 hours at 29°C in the present experiments. It is clear that the rate of increase in amino nitrogen observed here at the lower temperature does not differ from the rate observed by Ingle et al. by more than can be accounted for by the difference between the expected rates at 27° and 29°. The differences in rates previously reported are not the results of differences in techniques, but are most probably solely the result of differences in the temperatures of the animals during the periods of observation.

In the previous publication the effects on the blood amino nitro-

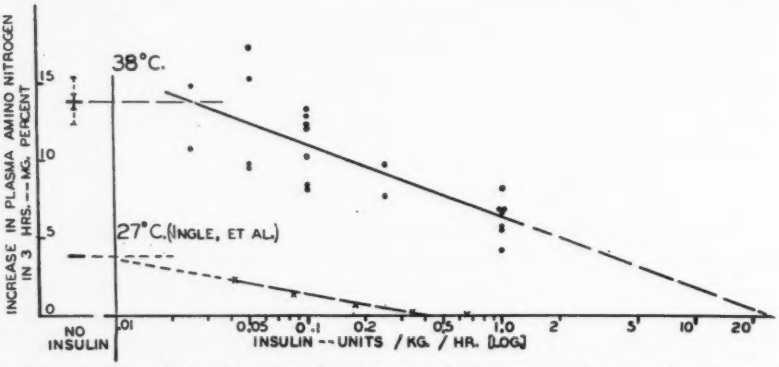

FIG. 1. The relationship of insulin dosage to the increase in plasma amino nitrogen in the eviscerated rat.

The average increase without insulin at 38° C. is given with its standard error and the standard deviation of the series. The slope of the solid line is that of the regression coefficient calculated from the individual points, each taken from a single experiment, -4.5 ± 0.8. The average values indicated in the lower part of the figure were recalculated from the data of Ingle, Prestrud, and Nezamis.

gen of only one dose of insulin, 2 units per kg. per hour given intravenously to animals kept at 38°C, was described. Ingle, et al., reported the effects of a range of doses, 0.25 to 16 units per rat (250 gm.) per day, or 0.04 to 2.67 units per kg. per hour, in rats kept at 27°C. Where at the higher temperature, only partial suppression of the amino nitrogen rise was obtained with 2 units per kg., complete suppression was reported with less than 1 unit per kg. per hour at the lower temperature. In Figure 1 is presented a log.-dose response curve obtained in animals kept at 38°C, in which increases in plasma amino nitrogen are related to doses of insulin from 0.025 to 1.0 unit per kg. per hour. For comparison, data taken from the figures of Ingle, et al., from animals kept at 27°C, are also presented. The slopes of the two lines and the positions with respect to dosage are similar, so that the absolute depression of the amino nitrogen rise induced by

any given dose of insulin is of the same order at the two temperatures. To reach the same degree of repression, such as 50 per cent inhibition, however, something like 10 times as much insulin would be required at the higher temperatures, and to prevent any increase at all, on the order of 25 units per kg. per hour would appear to be required, as compared to the 0.67 units observed by Ingle, et al., to be fully effective at the lower temperature.

As shown in Table 1, in confirmation of Ingle, et al., when the animals were maintained below 29°C, 1 unit insulin per kg. per hour was found to repress nearly completely the increase in amino nitrogen which otherwise occurred. This observation indicates again no essential difference between the results of Ingle, et al., and those of Frame and Russell except those due to temperature.

From the data summarized in Table 1, curves were constructed

TABLE 1. EFFECT OF TEMPERATURE AND INSULIN ON THE INCREASE IN PLASMA AMINO NITROGEN IN EVISCERATED RATS

	Increase in plasma amino nitrogen in 3 hours (1st to 4th hour after operation)			
	At 29°C		At 38°C	
	No. obs.	mg. per cent increase	No. obs.	mg. per cent increase
1. No insulin	7	5.9±0.41*	11	13.8±0.49
2. Insulin, 0.1 unit per kg. per hour (amorphous)			7	11.0±0.79
3. Insulin, 1 unit per kg. per hour				
a. Amorphous insulin	7	0.8±0.43	7	6.2±0.42
b. Zn-crystalline insulin free of glycogenolytic factor			6	4.5±0.84

* Standard error.

relating the natural logarithm of the amino nitrogen increase to temperature in the presence of insulin, as well as in its absence (Figure 2). Another group of experiments not shown in Table 1 was also included; here, when the body temperatures were maintained at 33°C, the average increase in plasma amino nitrogen in 3 hours was 1.76±0.53 mg. per cent. The slope of the line in the presence of insulin was 25.4±6.5 per cent per degree centrigrade, significantly greater than the figure of 9 per cent seen in the absence of insulin ($t=2.54$, with 35 degrees of freedom; $p<0.02$). The Q_{10} in the presence of insulin was approximately 7 fold. There is a suggestion here that the presence of insulin alters the reactions which limit the rate of protein breakdown. The data obtained with insulin are insufficiently exact, however, to allow any further characterization of the reactions affected.

It may be noted that although at 38°C exceedingly large amounts of insulin would be required to suppress completely the changes in blood amino nitrogen which occur after evisceration, a significant degree of depression may be obtained under these conditions with

doses of insulin which may be considered physiological. With 0.1 unit insulin per kg. per hour, the average difference of the increase in amino nitrogen from that seen in untreated animals was -2.8 ± 0.88 mg. per cent,[3] or about a 20 per cent diminution in rate of release of amino acids. The decrease observed with 1 unit of insulin per kg. per hour was of the order of 55 per cent (Table 1).

While the major observations reported here were made over the

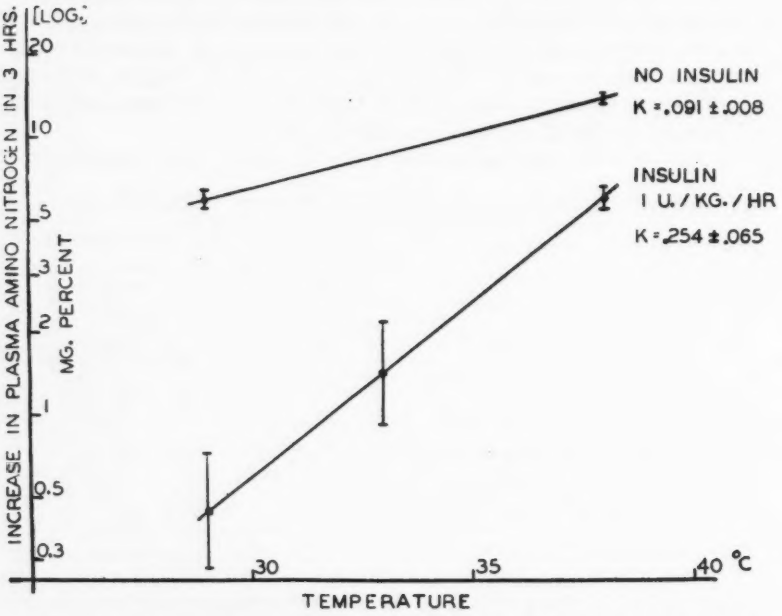

FIG. 2. The relationship of temperature on the rate of increase in plasma amino nitrogen in the eviscerated rat.

Mean values for each point, their standard errors, and the slopes of the lines (K) were calculated from the individual figures translated to logarithms. K, given in natural log. units, is the fractional increase in rate per °C. from the equation: $v = Ae^{Kt}$ or $\ln v = A' + K\ t$, where v = velocity and t = temperature. (The relationship of K to the Arrhenius coefficient or "temperature characteristic," μ, is: $K = \mu / R\ T^2$).

interval between 1 and 4 hours after evisceration, the effect of insulin on the plasma amino nitrogen was in fact observable at an earlier time. This may be seen in a comparison of the initial plasma amino nitrogen concentrations, determined 1 hour after evisceration and 30 to 40 minutes after the first administration of insulin. For the controls, receiving no insulin, this value was 10.0 ± 0.4 mg. per cent, while for the animals given 1 unit of amorphous insulin it was 6.9 ± 0.36 mg. per cent.

[3] $t = 3.2$, with 16 degrees of freedom; $p < .01$.

A sample of insulin stated to be virtually free of the glycogenolytic factor commonly found in insulin preparations[4] was also tested for its ability to diminish the extent of the increase in plasma amino nitrogen after evisceration. As shown in Table 1, there was no significant difference between the effects of this preparation and those of the usual amorphous insulin.

SUMMARY

The amino nitrogen content of the plasma of eviscerated rats maintained at 38°C increased an average of 14 mgm. per cent in 3 hours (between the 1st and 4th hours after operation). When the body temperatures of the rats were kept at about 29°C, the increase was reduced to 6 mgm. per cent. The calculated change in the rate of increase was then +9 per cent per °C increase in temperature. These figures indicate no essential difference between the results previously published by Frame and Russell and by Ingle, Prestrud and Nazemis, except those due to differences in the temperatures of the animals in the two series of experiments.

In eviscerated rats with body temperatures at 38°C, 1 unit of insulin per kg. per hour depressed the rate of increase in plasma amino nitrogen about 55 per cent, and 0.1 unit about 20 per cent. A log.-dose response curve relating insulin dosage to the increase in plasma nitrogen at 38°C is presented. At 29°C, 1 unit of insulin per kg. per kg. per hour prevented nearly completely the usual increase in plasma amino nitrogen. From 10 to 50 times as much insulin appear to be required to obtain the same relative effect on the plasma amino nitrogen at body temperatures of 38°C as at temperatures below 30°C.

Insulin freed of the pancreatic glycogenolytic factor was equally as effective as ordinary insulin in inhibiting the release of amino acids from the tissues.

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[4] This insulin (sample No. T2344, 26 units per mg.) was kindly furnished to Dr. Joseph Sokal by Dr. E. U. Campbell of the Lilly Company.

THE INTERVAL BETWEEN NORMAL RELEASE OF OVULATING HORMONE AND OVULATION IN THE DOMESTIC HEN[1]

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INTRODUCTION

THE RELEASE of ovulating hormone in the chicken (as well as in certain other spontaneously ovulating forms) may be conceived of as taking place either as an instantaneous discharge from the pituitary, or as a slow discharge over a period of one to several hours. Although we are inclined to the view that release in the chicken is more of an instantaneous than a slow discharge, we are unable to say now that it takes place definitely in one way or the other. Because of this, the term *release* in this and the accompanying paper (Rothchild and Fraps 1949) is used with certain reservations. By it is meant that point in the process of ovulation at which an amount of ovulating hormone adequate to cause ovulation is present in the blood stream, regardless of whether this amount left the pituitary immediately or over a period of some hours before.

Data on the time of release of ovulating hormone in the chicken are important, for the following reason. Enough information has been accumulated about the reproductive cycle in the chicken (Atwood, 1929; Warren and Scott, 1935; Phillips and Warren, 1937; Heywang, 1938; Warren and Scott, 1936; Byerly and Moore, 1941; McNally, 1946; Fraps, Neher, and Rothchild, 1947) to show an obvious relationship between daily cyclic changes in certain environmental factors and the characteristic rhythms of ovulation and lay. Inasmuch as there is a relationship between time of ovulation and environmental factors, the influence of the latter must undoubtedly be greater on the beginning of the process, i.e., release of ovulating hormone, than on the end of it, ovulation itself. Knowledge of the time of release, even partial knowledge, is thus helpful in the interpretation of the role that environmental factors play in the control or modification of the chicken's reproductive cycle.

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[1] This study is an enlargement of a preliminary one reported on at the 43rd annual meeting of the American Society of Zoologists (Rothchild, 1946). It is part of a study on the nature of the pituitary control over the processes of follicular maturation and ovulation in the domestic hen.

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The main features of the ovulation and laying cycles. (The authors cited in the preceding paragraph should be referred to for details.) The most obvious general feature of the reproductive cycle is the lay of eggs on successive days, each egg being laid at a successively later hour on each day than the one preceding it. The whole sequence of eggs is called a clutch. The initial egg of the clutch is usually laid before noon, the majority of successive intra-clutch eggs close to noon, while the terminal egg of the clutch is laid late in the afternoon. On the second day following that on which the terminal egg was laid, an egg laid comparatively early in the day initiates a new clutch. Clutches may vary in length from one to more than a dozen eggs, but in general, clutches of the same length follow one another in any particular bird.

The ovulation of each egg antedates its time of lay by an interval of roughly 24 to 28 hours. Successive ovulations (C_s ovulations) within a clutch, including the terminal ovulation (C_t ovulation), occur within an hour (average 30 minutes) following lay of the preceding egg. The initial ovulation (C_1 ovulation) of a clutch, however, always occurs at a comparatively early hour of the day (about 5:00 A.M.), about 18 hours or 18 hours plus some multiple of 24 hours following the lay of the terminal egg of the preceding clutch.

METHODS AND MATERIALS

Basis of the experimental procedure used. Hypophysectomy was performed at various intervals before an expected ovulation. If the expected ovulation actually occurred following the operation, it was assumed that release of the ovulating hormone had taken place at some time prior to the operation; conversely, if it did not occur, the assumption was that the complete release of hormone, or release of an amount adequate for ovulation, was expected at some time after the time of operation. The intervals between hypophysectomy and actual or expected ovulation were then calculated, and the relationship between these intervals and the percentage of birds ovulating was used as the basis for estimating the interval between assumed time of release of hormone and ovulation. A possible alternative interpretation of the results obtained by this method is given in the discussion of the paper accompanying this one (Rothchild and Fraps, 1949).

Determination of actual or expected ovulation time. Time of actual ovulation was estimated from the position of the resultant egg in the oviduct at the time of autopsy. For eggs found in the magnum or isthmus of the oviduct, a curve relating proportion of the oviduct traveled to time after ovulation was used (Warren and Scott, 1935; unpublished data from this laboratory). For eggs found in the uterus, a curve relating increase in weight of the albumen to time in the uterus was used (Burmeister, 1940). For eggs found broken in the body cavity, the same procedure was used that served to estimate the time of expected ovulation in the birds that failed to ovulate.

Time of expected C_1 ovulation in birds that failed to ovulate was estimated by means of an average interval between ovulation and oviposition of the C_1 egg. This interval varies as follows in relation to

clutch length: 1 egg clutches—29 hours; 2 egg clutches—27.5 hours; clutches of 3 eggs or more—27 hours (unpublished data from this laboratory). In the series of birds used in this study, the expected time of oviposition of the egg that failed to ovulate was assumed to be the same as the time of oviposition of the last C_1 egg that was actually laid; the time of expected ovulation was then determined by subtraction of the appropriate ovulation-oviposition interval from this time of estimated oviposition. An example may serve to clarify this. A bird that had just laid out a 3 egg clutch was hypophysectomized at 10:00 P.M. following the lay of the last egg of the clutch, and on autopsy the next day was found to have failed to ovulate. The time of the lay of the first egg of the clutch that preceded hypophysectomy was 8:00 A.M. The time of expected lay of the first egg of the succeeding clutch, whose ovulation had been prevented by hypophysectomy, was therefore assumed to be 8:00 A.M. Since the last clutch was 3 eggs in length, 27 hours were subtracted from 8:00 A.M. giving a time of expected ovulation of 5:00 A.M.

A simpler procedure was used to estimate the time of expected ovulation of C_s follicles that failed to ovulate. A C_s ovulation, as mentioned above, follows a preceding oviposition by an average interval of 30 minutes. The time of expected ovulation was therefore taken as 30 minutes from either a) the actual time of lay of the oviducal egg present at the time of hypophysectomy in those birds that laid the oviducal egg at the expected normal time, or b) the time of expected normal lay of the oviducal egg in those birds that showed a delay or other alteration from the normal in the lay of this egg (as some birds do following hypophysectomy—Rothchild, 1946).

General procedures and materials. The birds were regularly laying adults less than 2 years old, of the Rhode Island Red and White Leghorn breeds mainly, but with a few crossbreds included. They were maintained in individual cages in laying batteries on a 14 hour light day (onset of light at 6:00 A.M. EST), and allowed *ad libitum* access to a standard laying mash and water. The laying record preceding hypophysectomy was known for each bird used, since egg collections are recorded on the hour every hour from 8:00 A.M. to 4:00 P.M. and once again at 8:00 P.M. daily, as a routine laboratory procedure.

Each bird was allowed to remain undisturbed in its cage until the actual time of operation. Hypophysectomy was performed in the majority of cases by the transbuccal method described by Hill and Parkes (1934) and in the remainder by a modification of this technique developed by one of us (Rothchild, 1948). Following operation, which in no case required more than 15 minutes, and in almost all less than 10 minutes, the birds were returned to their cages under exactly the same conditions that prevailed before operation. Autopsies were performed 4 to 16 hours after hypophysectomy, the time of autopsy for each bird being such as to permit the optimum interval to elapse after

the time of expected ovulation in those birds that had apparently failed to ovulate and a minimum interval after ovulation in those birds that ovulated.

Birds found at autopsy to have been incompletely hypophysectomized were not included in the results if ovulation had occurred, but were included if ovulation had failed.

Preliminary runs demonstrated that failure of C_1 ovulation was uniform when hypophysectomy was performed before 10:00 P.M., but that almost all expected C_1 ovulations occurred when hypophysectomy took place after 2:00 A.M. All the data on the effect of hypophysectomy on the expected C_1 ovulation, therefore, were gathered from birds operated on between 10:00 P.M. and 2:00 A.M. In the birds hypophysectomized before an expected C_8 ovulation, the information gained from the studies on the C_1 ovulation served as a basis for adjusting the time of operation to each bird's expected time of ovulation, in order to obtain intervals between hypophysectomy and expected ovulation that were comparable with those in the C_1 group.

The results are presented in terms of the interval from hypophysectomy to actual or expected ovulation. To show them as simply but as accurately as possible, the birds were divided into three general groups: those showing (1) intervals of 4 hours or less; (2) intervals of more than 4 hours but less than 6 hours; and (3) intervals of 6 hours or more. The average interval in each group was calculated for the total group, and for the ovulating and non-ovulating birds within the group. (The average intervals shown for each group of birds in Tables 1 and 2 are valid cross-sections of each group, since the ovulating and nonovulating birds in each group showed almost identical average intervals.)

RESULTS

Hypophysectomy before an expected C_1 ovulation. The pituitaries were removed from 119 birds between 10:00 P.M. and 2:00 A.M. With the exception of 2 birds, the times of actual ovulation ranged from 3:00 A.M. to 6:45 A.M. and times of expected ovulation in the non-ovulating birds from 4:00 A.M. to 9:20 A.M. The two exceptions noted apparently ovulated at 1:30 A.M. and 12:00 noon, respectively. Intervals between hypophysectomy and actual or expected ovulation ranged from 10.5 hours in the bird ovulating at noon to 2.0 hours in the bird ovulating at 1:30 A.M. Except for these two birds, the range was from 9.33 hours to 3.0 hours.

The relationship between the percentage of birds ovulating and the interval from hypophysectomy to actual or expected ovulation is shown in Table 1. These results indicate that the most frequent interval between apparent release of hormone and ovulation is between 4 and 6 hours.

Hypophysectomy before an expected C_8 ovulation. The pituitaries were removed from 41 birds between 2:45 A.M. and 6:30 A.M. Times

TABLE 1.— RELATION BETWEEN THE INTERVAL FROM HYPOPHYSECTOMY TO ACTUAL OR EXPECTED OVULATION AND THE PERCENTAGE OF BIRDS OVULATING (SEE TEXT).

Interval: Hypophysectomy to actual or expected ovulations			Total number of birds		Birds ovulating			
	Average				Number		Per cent	
Range	C_1 ovulations	C_s ovulations	C_1 ovulations	C_s ovulations	C_1 ovulations	C_s ovulations	C_1 ovulations	C_s ovulations
(hours)	(hours)							
2.0– 4.0	3.3	3.5	10	11	10	9	100	82
4.2– 5.8	5.0	5.1	35	12	21	7	60	58
6.0–10.5	7.2	7.3	74	18	13	4	18	22

of actual ovulation ranged from 7:10 A.M. to 11:25 A.M. and times of expected ovulation in the non-ovulating birds from 8:30 A.M. to 12:30 P.M. The intervals between hypophysectomy and actual or expected ovulation ranged from 8.5 to 2.7 hours. The relationship between the percentage of birds ovulating and the interval between hypophysectomy and actual or expected ovulation is also shown in Table 1. There is evidently no appreciable difference between C_1 and C_s ovulations in regard to the interval between release of hormone and ovulation.

Of the 41 birds in the C_s ovulation group, 25 were expected to ovulate the terminal member of the clutch. The intervals from hypophysectomy to actual or expected *terminal* ovulations were compared with the intervals from hypophysectomy to all other actual or expected C_s ovulations. The numbers of birds falling into either

TABLE 2. COMPARISON OF THE RELATIONSHIP BETWEEN THE PERCENTAGE OF BIRDS OVULATING AND THE INTERVAL FROM HYPOPHYSECTOMY TO ACTUAL OR EXPECTED *terminal* OR *intraclutch ovulation*.

Interval: Hypophysectomy to actual or expected ovulation			Total number of birds		Birds ovulating			
	Average				Number		Per cent	
Range	terminal ovulations	intraclutch ovulations	terminal ovulations	intraclutch ovulations	terminal ovulations	intraclutch ovulations	terminal ovulations	intraclutch ovulations
(hours)	(hours)	(hours)						
2.7–5.0	4.0	3.7	9	8	6	8	68	100
5.5–8.5	6.75	7.0	16	8	4	2	25	25

group were not very large and therefore only two ranges of intervals were used: those of 5 hours or less, and those of more than 5 hours. The results are shown in Table 2.

They indicate a possibility (no more than slight, considering the number of birds involved) that if anything, the interval from release of hormone to a terminal ovulation is slightly less than it is for all other ovulations in the clutch.

DISCUSSION

The results presented in this paper demonstrate fairly well that the interval between release of ovulating hormone and ovulation is practically the same for all members of a clutch. The successively later hours at which ovulations occur within a clutch are thus traceable to successively later releases of ovulating hormone. Thus, since ovulations occur only within a circumscribed portion of the solar day, the releases of hormone must occur within an equally circumscribed period of time. The question of why releases of ovulating hormone occur only within certain time limits resolves itself into three interdependent questions, namely—what changes in the environment and in the animal body are associated with the stimulus that leads to the earliest release of ovulating hormone? What changes occur each day that lead to releases of the ovulating hormone at successively later hours each day? And, what changes in the environment and in the animal body are associated with the failure or prevention of a release on the day of oviposition of the terminal egg of the clutch?

These questions are raised, not because they can be answered now, but in order to define in clear terms the problem we are faced with—that of determining the nature of the relationship between the environment in which the chicken lives, and its reproductive behavior. The first steps in this direction were made by the authors cited above. The present study is an additional step forward but no means a large one. From other work (Fraps, Neher and Rothchild, 1947) taken together with this one, we now know that the *general limits* of time within which the releases of hormone occur approximate those in which the dark hours or periods of non-feeding occur, and in which the body temperature of the chicken is in general below the daily average and probably restricted to the ascending portion of the body temperature curve. But to define more exactly the temporal boundaries between earliest and latest possible time of release of ovulating hormone, it is necessary to know the extent to which the method used here measures the *actual* time of release of ovulating hormone. This point is taken up briefly in the discussion of the paper accompanying this one, (Rothchild and Fraps, 1949).

SUMMARY

Mature, regularly laying domestic hens were hypophysectomized at intervals ranging from 10 hours to 2 hours before an expected ovulation. The expected ovulation in 119 cases was that of an initial member of a clutch, and in 41 cases was that of an intraclutch or terminal member. It was found that the percentage of birds ovulating the expected ovum increased with the decrease in the interval from hypophysectomy to actual or expected ovulation, and that the most frequent interval between apparent release and ovulation was of the order of 4 to 6 hours, and was not related to position in the clutch.

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THE INDUCTION OF OVULATING HORMONE RELEASE FROM THE PITUITARY OF THE DOMESTIC HEN BY MEANS OF PROGESTERONE[1]

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INTRODUCTION

THE INDUCTION of ovulation in the hen by means of progesterone was first noted by Fraps and Dury (1943). Later, unpublished experiments of Fraps and his coworkers indicated that the probable cite of action of this hormone was the bird's own pituitary. These experiments showed (1) that the ovarian follicles of hens that had been treated with pregnant mare's serum could not be ovulated with progesterone, but were capable of being ovulated with luteinizing pituitary substances; and (2) that the pituitaries of these hens treated with pregnant mare's serum were considerably lower in ovulating potency than the pituitaries of normal laying hens.

The present studies were undertaken to determine as conclusively as possible whether or not progesterone induced a release[3] of ovulating hormone from the pituitary of the treated hen, and to what extent this release resembled the natural one. Because of the well known ovulation-inhibiting effect of progesterone in mammals, the question of progesterone inhibition of ovulation in chickens was also considered briefly.

METHODS AND MATERIALS

The ovulation and egg-laying pattern of the domestic hen is described in sufficient detail in the first paper in this series (Rothchild and Fraps, 1949), for an understanding of the plan of these experiments. The test ovarian follicle in all the birds used in this study was the C_1 or first follicle of an oncoming clutch. This follicle ovulates normally at about 5:00 A.M. under the conditions in this laboratory, but injections of progesterone at any time after about 1:00 P.M. on the day preceding its expected ovulation induce it to

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[1] A preliminary report of these studies was made at the 31st annual meeting of the American Physiological Society (Rothchild and Fraps, 1947).

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[3] See introduction to first paper in this series (Rothchild and Fraps, 1949) for limitations on the use of this term.

ovulate at an average interval of 9.0 hours from the time of injection (see below). The normal ovulation of this follicle can be prevented uniformly by hypophysectomy carried out at 10:00 P.M. (Rothchild and Fraps 1949) or earlier on the day preceding its expected ovulation. In the present studies, except where otherwise noted, all injections took place between 1:00 P.M. and 7:30 P.M. and all operations (complete or sham hypophysectomies) between 2:20 P.M. and 10:00 P.M. on the day preceding the expected C_1 ovulation.

Progesterone was dissolved in propylene glycol at a concentration of 10.0 mg./ml. for intravenous use, and in corn oil at a concentration of either 10.0 mg./ml. or 12.5 mg./ml. for subcutaneous use.

The pituitary material used was a preparation of male chicken anterior pituitaries, frozen immediately after collection and dried on the same day by pressing them out on glass plates, and exposing the plates to a current of warm air. The dried powdered material was stored in a desiccator over $CaCl_2$. For use it was taken up in distilled water and injected intravenously in a volume of 0.10 to 0.20 ml. per bird (0.5 mg./ml. or 1.0 mg./ml.).

The operative technique used was hypophysectomy. It was performed in most cases by the transbuccal method described in detail by Hill and Parkes (1934). A modification of this technique developed by one of us (Rothchild, 1948) was used on a small number of birds added after the main features of the study had been carried out with the older method. Sham hypophysectomy consisted of every step of the transbuccal operation except the removal of the pituitary.

Autopsies, except where otherwise noted, took place within 10 to 18 hours from the time of hypophysectomy. The occurrence and time of actual ovulation were determined as described before (Rothchild and Fraps, 1949), the Burmeister (1940) curve[4] being used in the majority of cases. Time of expected ovulation in the non-ovulating birds and in the ovulating birds in which the yolk was found broken in the body cavity was estimated to be 9.0 hours from the time of injection of progesterone, since this was the average interval (range: 6.0–11.3 hours; most frequent interval: 8.0–10.0 hours) between injection and ovulation[4].

Incompletely hypophysectomized birds were excluded from the results if ovulation occurred, but were included if ovulation had failed.

Breeds, ages and conditions of maintenance were the same as described in the first paper of this series (Rothchild and Fraps, 1949).

EXPERIMENTS AND RESULTS

Experiments 1, 2 and 3 deal with the mechanism of the ovulation-inducing action of progesterone in the domestic hen; experiment 4 with the ovulation-inhibiting action of progesterone.

Experiment 1 was set up to determine whether progesterone would induce ovulation in the absence of the pituitary. The plan and results

[4] Use of the Burmeister curve would presumably involve a greater error in determination of ovulation time than the use of the method involving the location of the egg in the magnum. However, in 12 control birds, ovulation time as determined by the latter method averaged 8.33 hours from time of injection of progesterone—a value which is certainly of the same order as that determined by means of the Burmeister curve.

of the experiment are presented in Table 1. These demonstrated very clearly that the absence of the pituitary completely abolished the ovulation-inducing action of progesterone.

TABLE 1. FAILURE OF PROGESTERONE TO INDUCE OVULATION IN THE HYPOPHYSECTOMIZED HEN. THE OVULATIONS NOTED OCCURRED ROUGHLY 9 HOURS AFTER THE INJECTION OF PROGESTERONE, OR ABOUT 6 HOURS BEFORE THE EXPECTED NORMAL TIME.

Progesterone dose per bird	Route of administration	Time of hypophysectomy	Birds treated	Birds ovulating	
(mg.)			(no.)	(no.)	(%)
1.0	intravenous	unoperated on	30	25	83
10.0	intravenous	unoperated on	7	7	100
10.0	intravenous	immediately before injection	10	0	0
10.0	intravenous	2 hours after injection	11	0	0
10.0	subcutaneous[1]	2 hours after injection	5	0	0
1.0	intravenous	*sham hypophysectomy* immediately before injection	11	11	100

[1] No controls were run on this level of progesterone by this route of administration but in Fraps and Dury's paper (1943) between 90%–100% of birds ovulated a C_1 follicle in response to subcutaneous injection of 1.0–10.0 mg. of progesterone.

Experiment 2 was set up to determine whether the ovarian follicle was capable of ovulating in the absence of the pituitary. The experimental plan and results are shown in Table 2. Although a decrease in responsiveness of the follicle occurred immediately after hypophysectomy, it was still able to ovulate in response to sufficiently large doses of pituitary powder, a result quite different from that obtained with progesterone.

TABLE 2. ABILITY OF THE OVARIAN FOLLICLE TO OVULATE FOLLOWING HYPOPHYSECTOMY.

Dose of pituitary[1] per bird[2]	Time of hypophysectomy	Birds treated	Birds ovulating	
(mg.)		(no.)	(no.)	(%)
0.05	unoperated on	35	28	80
0.05	immediately before injection	19	2	10
0.10	immediately before injection	21	11	52
0.20	immediately before injection	10	8	80

[1] See Methods and Materials.
[2] All injections were made intravenously in this series of birds.

The two experiments taken together indicated that the failure of ovulation in the hypophysectomized bird following progesterone injection was most probably due to a lack of pituitary action on the follicle and, therefore, that progesterone in all likelihood induced ovulation by stimulating a release of ovulating hormone from the pituitary.

Experiment 3 was carried out (a) to determine the time required

for the completion of the assumed release of ovulating hormone following progesterone injection, and (b) to compare (1) the interval from time of assumed release induced with progesterone to ovulation with (2) the interval from the time of natural release to ovulation.

Progesterone was injected intravenously, the pituitaries removed up to and including 4.0 hours afterwards, and the incidence and time of ovulation noted. In Table 3 the incidence of ovulation in relation to the interval from injection to hypophysectomy is summarized; evidently the pituitary must be left in situ for 4 hours following the injection of progesterone for a maximum normal ovulating response to take place. The significance of this is taken up in the discussion.

TABLE 3. RELATION BETWEEN THE INTERVAL FROM PROGESTERONE INJECTION (INTRAVENOUS) TO HYPOPHYSECTOMY AND THE INCIDENCE OF OVULATIONS

Time from progesterone injection to hypophysectomy	Amount progesterone injected	Birds treated	Birds ovulating	
(hours)	(mg.)	(no.)	(no.)	(%)
0—less than 2[1]	1.0	16	0	0
	10.0	10	0	
2	1.0	11	2	9
	10.0	11	0	
More than 2 to less than 3[2]	1.0	21	8	38
3	1.0	15	10	67
4	1.0	17	14	82

[1] Hypophysectomy immediately before progesterone injection to 101 minutes after.
[2] Hypophysectomy between 132 and 166 minutes after progesterone injection (average—150 minutes).

Table 4 shows the relationship between the interval from hypophysectomy to expected or actual ovulation and the percentage of birds ovulating in response to progesterone, in comparison with the same relationship in birds ovulating under natural conditions. The time relations involved are practically identical for both naturally-induced

TABLE 4. NATURALLY-OCCURRING AND PROGESTERONE-INDUCED OVULATIONS COMPARED FOR THE RELATIONSHIP BETWEEN THE PERCENTAGE OF BIRDS OVULATING AND THE INTERVAL FROM HYPOPHYSECTOMY TO ACTUAL OR EXPECTED OVULATION.

Interval: Hypophysectomy to actual/expected ovulation			Total number of birds		Birds ovulating			
Range	Average				Number		Percent	
	Natural[1]	Progesterone[2]	Natural[1]	Progesterone[2]	Natural[1]	Progesterone[2]	Natural[1]	Progesterone[2]
(hours)	(hours)	(hours)						
2.0– 4.0	3.5	3.3	21	5	19	5	90	100
4.2– 5.9	5.1	5.3	47	21	28	15	60	71
6.0–10.5	7.2	7.3	92	77	17	16	18	21

[1] Ovulations occurring under natural conditions, i.e., normally expected C_1 and C_2 ovulations together (see Rothchild and Fraps, 1949).
[2] Ovulations induced with progesterone.

and progesterone-induced assumed releases of ovulating hormone.

Experiment 4, as indicated above, involves the inhibition of ovulation with progesterone. It has been known for some time in this laboratory that although progesterone will induce ovulation, at least the oldest follicle in some birds that fail to ovulate, and some or all of the younger follicles in those birds in which ovulation occurs, will undergo atresia. This effect takes place even with dosages of progesterone as low as a fraction of a milligram. The action of progesterone on follicles *less mature* than the ovulable one is thus evidently primarily an ovulation-inhibiting one.

To demonstrate this even more conclusively, 15 birds were selected for particularly regular lay records, and at 4:00 P.M. on the day of ovulation of a terminal follicle, i.e., roughly 37 hours before the next expected ovulation, subcutaneous injections of progesterone were begun. A total dose of 12.5 mg. of progesterone per bird per day was given, divided into two injections, at 4:00 P.M. and 8:00 A.M. In 10 birds the injections were continued until 4:00 P.M. of the day of expected C_1 ovulation, and the birds were killed at 8:00 A.M. the next day, roughly 28 hours after the expected ovulation. The remaining 5 birds were killed exactly 24 hours earlier than this, roughly 3 hours after the time of expected ovulation. In all 15 birds ovulation failed to occur. In 5 of the 10 birds killed 28 hours after expected ovulation, and in 3 of the 5 birds killed 3 hours after expected ovulation, the follicles were in atresia.

Although this experiment is admittedly crude, it demonstrates the difference in the effect of progesterone when injected at different intervals before an expected ovulation. The same *level* of progesterone, or one slightly less than this (10.0 mg.) when injected by either the subcutaneous or intravenous route on the day *after* the ovulation of the terminal follicle has primarily an ovulation-inducing effect.

In another connection, this same relationship was demonstrated in the laboratory recently by Mr. B. H. Neher. He injected a total of either 2.0, 4.0 or 6.0 mg. of progesterone (using 1, 2 or 3 injections of 2.0 mg. each) into 3 groups of birds. In 16 birds a single injection was made at 4:00 P.M. on the day of ovulation of the terminal egg; in 12 of these birds, a second injection was made at 12:00 midnight following the first injection; and in 8 of these birds a third injection was made at 9:00 A.M. the following day. No autopsies were carried out, but the lay records of these birds indicated an indefinite delay in ovulation or atresia (more likely the latter) of the C_1 follicle in 3 of the 4 birds given the single, 3 of the 4 birds given the double, and 7 of the 8 birds given the triple injection. It will be noted that the first injection in every case took place at 4:00 P.M. following the terminal ovulation. However, in another group of birds, he made a single injection at 4:00 A.M. following the terminal ovulation and in these birds the effect was not inhibition of ovulation but an induction of ovulation.

DISCUSSION

The facts concerning the ovulation-inducing action of progesterone are these: ovulation cannot be induced with progesterone in birds whose pituitaries are low in ovulating potency (as shown by the pregnant mare's serum experiments of Fraps, et al. cited above) or in hypophysectomized birds, but progesterone does induce ovulation in normal or sham hypophysectomized birds; ovulation can be induced in birds whose pituitaries are either lacking or low in potency, by the injection of pituitary substance; the percentage of birds ovulating following progesterone injection is zero when the pituitary is removed within less than 2 hours after injection, but rises to the same value shown by normal birds when the pituitary is removed at 4.0 hours after the injection; the percentage of birds ovulating in relation to the interval from hypophysectomy to expected ovulation is practically the same for birds ovulating under natural conditions or in response to progesterone. These facts, we feel, justify the assumption that progesterone induces a release of ovulating hormone from the pituitary.

A comparison of the time relations between hypophysectomy and naturally induced ovulations, and hypophysectomy and progesterone-induced ovulations, forces on our consideration the question of whether or not the assumptions made in carrying out the experiments reported in the preceding paper were correct. These were: if hypophysectomy was followed by ovulation, the release occurred before the time of hypophysectomy; if hypophysectomy prevented ovulation the release was expected after the time of hypophysectomy. Yet the data reported in the present paper make it fairly clear (1) that progesterone stimulates a release of hormone from the pituitary, and (2) that hypophysectomy at any time within less than 4 hours following the injection of progesterone partially or completely prevents the expected ovulation. It seems rather unreasonable to assume, as an explanation of this, that the release of hormone occurs as late as 4 hours after the injection of progesterone, especially since intravenously injected progesterone probably has only a very short stay in the blood stream. What is perhaps a more likely explanation is the following. After a release of ovulating hormone, the continued secretion of a follicle-maintaining factor by the pituitary is necessary for the follicle to initiate its reaction to the ovulating hormone. The time before expected ovulation when this continued secretion becomes dispensable, then, would be the time at which hypophysectomy does not prevent ovulation, and it would follow the time of release of hormone by some amount up to 4 hours. The other side of the same coin would be, of course, that failure of ovulation following hypophysectomy could signify not only the possibility that hypophysectomy preceded the time of expected release, but also that it followed the actual release of hormone at some interval less than that required for complete mainte-

nance of the follicle in a state of reactivity to the ovulating hormone. (Some experiments in progress now indicate this as a worthwhile possibility, but since they are by no means conclusive as yet, the discussion of their significance must be left for a future date.)

As has already been mentioned (Fraps, Neher and Rothchild, 1947; Rothchild and Fraps, 1949) the release of the ovulating hormone under natural conditions takes place only within a circumscribed portion of each 24 hours. Since C_1 ovulation can be induced by injections of progesterone at times when naturally-induced releases of ovulating hormone do not occur, the pituitary is evidently responsive to a release stimulus at these times, and the failure of the natural release to occur may be due to a drop in or absence of activity of the normal release-provoking system at these hours of the day. It is also possible that the pituitary itself shows a cycle of responsiveness to the release stimulus, and that the failure of release at certain hours of the day is due to the fact that the pituitary has lost its responsiveness to a release-provoking stimulus. Since the action of progesterone so closely parallels that of the natural release stimulus, if it is not actually the stimulus itself, it may serve as a useful tool with which to demonstrate the existence and characteristics of such a cycle of responsiveness in the pituitary.

The belief that, in regard to ovulation, progesterone is primarily an ovulation-inhibiting hormone rests largely on the following experimental evidence. Removal of corpora lutea in the guinea pig is followed by a shortening of the estrus cycle (Loeb, 1911); the drop in ovulating potency of the estrus rabbit's pituitary that occurs following coitus does not take place following coitus of the pseudopregnant rabbit, nor does the pseudopregnant rabbit ovulate following coitus (Makepeace, Weinstein and Friedman, 1938); continued injections of crude extracts of corpora lutea or of progesterone interrupt the estrus cycles of rats and mice (Papanicalaou, 1926; Parkes and Bellerby, 1927; Hisaw, Meyer and Weichert, 1928; Haterius and Pfiffner, 1929; Patel, 1930; Selye, Browne and Collip, 1936; Phillips, 1937), prevent the induction of pseudopregnancy in rats following cervical stimulation (Astwood and Fevold, 1939), the preovulatory swelling of the Graafian follicles in the guinea pig (Dempsey, 1937) the ovulation that normally follows coitus (Makepeace, Weinstein and Friedman, 1937), or copper injections (Friedman, 1941) in the rabbit, and the luteinization of the ovary that usually follows injections of follicle stimulating hormone in the immature rat (Astwood and Fevold, 1939).

The injections of progesterone or progestin in most of these cited experiments were made in relatively large amounts, and over long periods of time. In the only experiment described in the literature in which progesterone was given in a single injection in a relatively high dose to a normal mammal, the effect of the progesterone was appar-

ently to induce an ovulation (Everett, 1944). The same author had previously shown that progesterone facilitated if it did not actually induce ovulations in constant estrus rats (Everett, 1940).[5]

In the chicken progesterone has either an ovulation-inhibiting or an ovulation-inducing effect depending on the time of administration. Practically all the evidence for progesterone's ovulation-inhibiting activity in the mammal is of the same order as that which demonstrated inhibition in the bird, i.e., injections were started relatively early in regard to time of expected ovulation, and continued for a relatively long period of time. These facts lead us to believe that the role of progesterone in the mammalian estrus cycle deserves reexamination.[5] The fact that Dempsey, Hertz, and Young (1936) found evidence for the secretion of progesterone in the guinea pig several hours before ovulation also contributes to the possibility that progesterone, in the follicular phase of the mammalian estrus cycle, may actually be involved in the induction of ovulation, while in the luteal phase one of its principal roles is that of inhibition of ovulation.

SUMMARY

An amount of progesterone 10 times that required to induce ovulation in intact normal birds was found to be completely ineffective when injected immediately after hypophysectomy; the dose required for normal birds, however, was completely effective when injected immediately after sham hypophysectomy. Removal of the pituitary within 2 hours after the injection of progesterone prevented ovulation from taking place. Hypophysectomy between 2 and 4 hours after injection permitted increasing percentages of birds to ovulate, the same percentage as normal ovulating when hypophysectomy was delayed until 4 hours after the injection. The maximum interval from hypophysectomy to actual or expected ovulation at which the maximum percentage of birds ovulated was of the order of 4 to 6 hours, which was the same value found for birds ovulating in response to a natural release of ovulating hormone. These facts justify the conclusion that progesterone induces a release of ovulating hormone from the pituitary of the chicken.

The ovulation-inhibiting effect of progesterone described for mammals was demonstrated in the chicken as well. Inhibition of ovulation in the latter follows progesterone injections made at intervals of 36 hours or greater before an expected ovulation.

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THE INFLUENCE OF GROWTH HORMONE ON FASTING METABOLISM[1]

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THE ROLE of the adrenal cortex and of the thyroid in regulating the rate of mobilization of nitrogen from the tissues of the fasting mouse has been investigated recently by White and Dougherty (1947). It was found that the adrenal cortical steroids affected the rate of loss of nitrogen from lymphoid tissue. Mobilization of nitrogen from the carcass of the fasting mouse was negligible in the absence of the thyroid, although nitrogen loss from the liver of either thyroidectomized or adrenalectomized animals remained relatively independent of these hormonal influences. The data further suggested that translocation of nitrogen from carcass to lymphoid tissue of the fasting animal was influenced by thyroid secretion.

These attempts at localization of endocrine control over the contribution of nitrogen from specific tissues in the fasting animal are extended in the present study to the investigation of another potent endocrine influence, namely, purified growth hormone. This has been done with a view to obtaining evidence regarding the locus of the nitrogen-sparing action of this hormone. Accordingly, the effect of purified adenohypophyseal growth hormone on the net changes of nitrogen, as well as water and lipid, in specific tissues was investigated in both intact and adrenalectomized mice during a 48-hour fast. It was found that this growth-promoting preparation decreased the rate of protein utilization and accelerated lipid metabolism of control and adrenalectomized mice.

In spite of its pronounced effects on liver and carcass composition and weight loss, purified growth hormone was without influence on the metabolism of lymphoid tissue of intact mice during fasting, except at very high dose levels where augmented fasting involution of lymphoid structures was observed. Administration of growth hormone to fasting, adrenalectomized mice failed to potentiate the growth

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of lymphoid tissue. These observations would appear to lend additional support to the specificity of pituitary-adrenal cortical control over lymphoid tissue weight, structure and function.

METHODS

Male mice of the CBA strain (Strong), between 8 to 10 weeks of age and weighing 18 to 22 grams, were employed. Preceding the fast, all animals were maintained on Purina laboratory chow pellets supplemented with powdered calf meal. The mice were kept in individual metabolism cages during fasting, with free access to water; the adrenalectomized animals received 0.9% sodium chloride as drinking fluid. Subcutaneous injection of 0.25 mgm. desoxycorticosterone acetate (in 0.05 ml. sesame oil, Schering) was used as an immediate postoperative supportive measure. Adrenalectomized mice were allowed food for 24 to 48 hours before institution of the fast.

Growth hormone[3] in physiological saline was injected intraperitoneally in a total dose of either 200 micrograms or 4 milligrams, respectively. The hormone was administered in 4 divided doses over the 48-hour fasting period. Control fasted animals were injected with saline.

Sampling and analytical procedures

At the end of the fasting period, the mice were sacrificed by severing the cervical vertebrae, and samples of tissue from individual animals were obtained immediately for analysis.

a. *Lymphoid tissue*

A representative and uniform sample of lymphoid tissue, consisting of paired axillary and inguinal nodes, six cervical nodes, the mesenteric node chain, thymus, and spleen, was rapidly dissected free of surrounding fat and connective tissue. Suitable precautions were taken to prevent tissue dehydration. The lymphoid tissue was weighed immediately in a small tared vial on the analytical balance, dried at 105°C. overnight, and a dry weight determined.

In the initial studies, the water-free samples were transferred quantitatively with the aid of ethanol to a semi-micro Soxhlet appratus. Total lipid

[3] The growth hormone preparation employed was made available through the courtesy of Drs. A. E. Wilhelmi, J. B. Fishman and J. A. Russell of the Department of Physiological Chemistry, Yale University. It was derived from beef anterior pituitary tissue and had been purified by repetition of the calcium hydroxide—ethanol fractionation procedure described by Fishman, Wilhelmi, and Russell (1947). When assayed by these investigators in 100 gm. hypophysectomized rats, 10 micrograms of this preparation administered daily for 10 days permitted an average total weight gain of approximately 9 gm. The increase in width of the epiphyseal cartilage in similar animals averaged 78 micra. Comparable material assayed at the Armour Laboratories in Chicago, through the courtesy of the late Dr. Fred C. Koch, showed no demonstrable lactogenic, gonadotrophic, or posterior lobe hormonal activity. The preparation contained less than 1 per cent of the adrenotrophic activity of a standard preparation of adrenotrophin, and 0.1 Evans chick unit of thyrotrophic potency per milligram. These hormonal contaminants did not appear to be significant factors in the experiments described in the present report. This may be deduced from the high activity of the preparation in the adrenalectomized animals, and from data obtained from histological examination of thyroids of fasted, growth hormone-treated mice. The latter glands were indistinguishable from those of control fasted animals in their resting, basal appearance.

was estimated gravimetrically by a modification of the procedure described by Roberts and Samuels (1946). This included exhaustive extraction of the sample with alcohol and ether successively, and resolution with petroleum ether. The dry, fat-free tissues were transferred quantitatively to a Kjeldahl flask and digested with 2 ml. of concentrated sulfuric acid in the presence of a Hengar granule as catalyst. Digestion was completed with 5 drops of Superoxol. Suitable aliquots were analyzed for nitrogen by the method of Sobel et. al. (1937), with the exception that mixed methyl red—bromcresol green was used as indicator. The nitrogen content of the petroleum ether-insoluble fraction of the total lipid extract was also determined (lipoprotein nitrogen), and added to the above value. Total nitrogen multiplied by the factor 6.25 was used as an approximation of the protein present.

The lymphoid tissue water, nitrogen and lipid concentrations under specific conditions were strikingly reproducible. These three values accounted for 100% of the tissue composition, with a maximum deviation of 1.5%. Therefore, in later experiments it was considered unnecessary to determine total lymphoid tissue lipid; this value was derived by difference, using the observed concentrations of water and nitrogen.

b. *Liver.*

At autopsy, a sample of liver was blotted free of blood and taken for water analysis, as described above. Duplicate fresh samples, weighed rapidly on the Roller-Smith torison balance, were obtained for total nitrogen determination. The remaining liver tissue was weighed and preserved in 95% ethanol for subsequent lipid analysis as described above; these samples were ground with reagent grade sea sand prior to lipid extraction, In some cases, samples of liver were placed in 10% formalin for subsequent histological examination.

c. *Carcass.*

Following dissection of the lymphoid tissue and liver, the gastrointestinal tract was removed, weighed and discarded. The remaining carcass was frozen rapidly in a mixture of diethyl ether and solid carbon dioxide. It was then ground rapidly to a fine uniform powder in a Quaker City laboratory power mill which had been cooled previously by grinding dry ice. Prior to grinding of the carcass, excess carbon dioxide was blown out of the mill with a stream of compressed air.

Weighed samples of ground carcass were then taken for water, nitrogen, and lipid analysis by methods described above. The aliquot for total lipid was stored in 95% ethanol, and subsequently transferred directly, without further grinding, to the Soxhlet apparatus.

RESULTS

1. *The effect of adrenalectomy on tissue composition of the fed animal.*

Intact and adrenalectomized mice were sacrificed in the fed state to provide data from which was inferred the tissue composition at the institution of the fast. Comparisons of the analytical data were made with regard to the appropriate controls: intact fasted with intact fed, and adrenalectomized fasted with adrenalectomized fed, animals.

The reason for the distinction in controls will be clear from Table 1

TABLE 1. CHANGE IN TISSUE COMPOSITION OF MALE CBA MICE 24–48 HOURS AFTER ADRENALECTOMY†

	INTACT		ADRENALECTOMIZED		
	Total present gm.	gm./100 gm.B.W.	Total present gm.	gm./100 gm.B.W.	% Change
Liver					
Protein	0.296	1.32	0.252	1.22	− 7.6
Water	0.955	4.26	0.792	3.84	− 9.8
Lipid	0.103	0.46	0.083	0.40	−13.0
Carcass					
Protein	4.41	19.7	4.08	19.8	+ 0.5
Water	14.90	66.5	13.90	67.5	+ 1.5
Lymphoid Tissue					
Protein	0.032	0.143	0.040	0.194	+35.7
Water	0.152	0.680	0.191	0.926	+36.2
Lipid	0.015[1]	0.067	0.011[2]	0.053	−20.9

[1] By analysis.
[2] By difference, using H_2O and N percentages observed.
† Numbers of animals used indicated in legend for Figure 1.

in which are tabulated the effects of adrenalectomy on the composition of lymphoid tissue, liver, and carcass of fed animals. It will be observed that loss of protein, water and lipid from the liver occurred in the adrenalectomized group. Total liver weight was thus significantly decreased in these animals with respect to the fed controls (see also Table 2). Carcass exhibited virtually no change.

TABLE 2. THE INFLUENCE OF GROWTH HORMONE ON LIVER WEIGHT IN MALE CBA MICE DURING FASTING†

Group	No. of Animals	Total Liver Wet wt. (gm/100 gm. B.W.)	Total Liver Wet wt. (gm.)	Total Liver wt.-Fat-free (gm.)	Total Liver wt.-dry, fat-free (gm.)
Intact					
Fed	8	6.07 ± 0.19	1.3621 ± 0.049	1.2592 ± 0.044	0.3025 ± 0.022
Fasted 48 hrs.	13	5.16 ± 0.16	0.8640 ± 0.038	0.7870 ± 0.030	0.2013 ± 0.010
Fasted 48 hours.+ Growth Hormone, 200 μgm.	14	6.36 ± 0.07*	1.1110 ± 0.044*	0.9240 ± 0.028*	0.2278 0.010
Fasted 48 hrs.+ Growth Hormone, 4 mgm.	6	6.92 ± 0.31*	1.2433 ± 0.070*	0.9481 ± 0.028*	0.2345 ± 0.014*
Adrenalectomized					
Fed	13	5.43 ± 0.11	1.1210 ± 0.039	1.0380 ± 0.038	0.2460 ± 0.008
Fasted 48 hrs. (+saline)	11	4.57 ± 0.10	0.8896 ± 0.025	0.8045 ± 0.025	0.1732 ± 0.006
Fasted 48 hrs.+ Growth Hormone, 200 μgm.	10	5.28 ± 0.12*	1.0300 ± 0.025*	0.8710 ± 0.026*	0.1910 ± 0.007*

† Deviations shown are standard errors.
* The starred values are significantly different from the respective fasted control figures.

The well-known hypertrophy of lymphoid structures following adrenalectomy was also observed in the present investigation (Table 1; see also Table 5). The composition of this growth appeared to be an increase in the amounts of water and protein present, accompanied by a reduction in total lipid (Table 1). The latter, however, may be of questionable significance, because the absolute amounts of lipid estimated were very small.

These data are illustrated graphically in Figure 1.

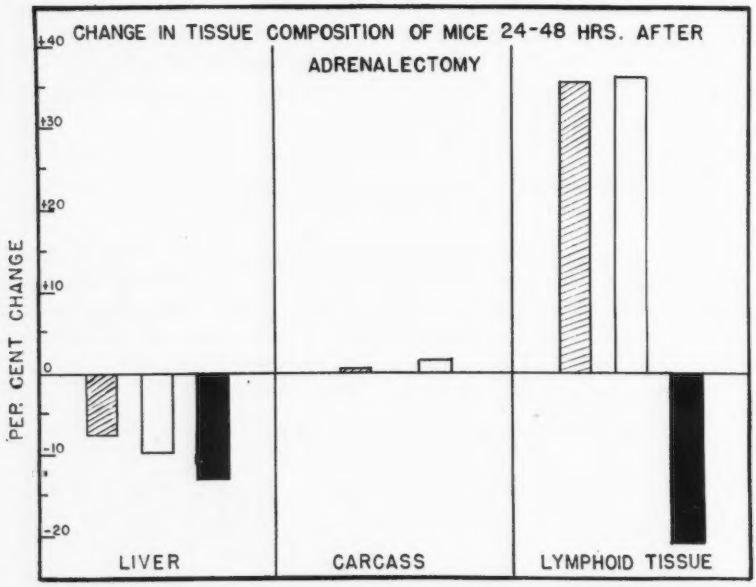

FIG. 1. The length of the bars represents the per cent change of tissue constituents in fed adrenalectomized animals, 24–48 hours after operation, related to the tissue composition of intact controls taken as a zero baseline. The cross-hatched bars represent change in protein, the open bars, change in water, and the solid bars, change in lipid. The number of individual animals contributing to the average values were 8 intact controls, 9 adrenalectomized animals used for lymphoid tissue and carcass analysis, and 13 similar animals for liver data.

2. *The influence of growth hormone on liver of fasting mice.*

a. *Weight and composition*

Intact mice fasted for 48 hours lost approximately 32% of liver protein when compared with fed controls (Figure 2). The degree of water loss was of a similar order of magnitude. If, however, growth hormone was administered during the fast, there was a statistically significant reduction in the degree of nitrogen and water loss from the liver, i.e., a relative retention of nitrogen, in the complete absence of exogenous sources of protein. This is seen Figure 2 for two doses of growth hormone in intact animals, and for the 200 microgram dose in

the adrenalectomized group. It will be noted that protein loss from the liver of adrenalectomized animals during fasting was very similar in magnitude to that observed in intact mice, as had been reported previously (cf. White and Dougherty, 1947). The ratio of water: protein loss, however, was different in the two groups.

The most striking effect of growth hormone seen in these studies was the intense mobilization of lipid to liver tissue. It may be noted in Figure 2 that although there was an actual loss of about 20% of the total lipid from the liver in the untreated intact group during the fast, this was counteracted to a striking degree by the administration of growth hormone. The scale here is 8 times reduced over

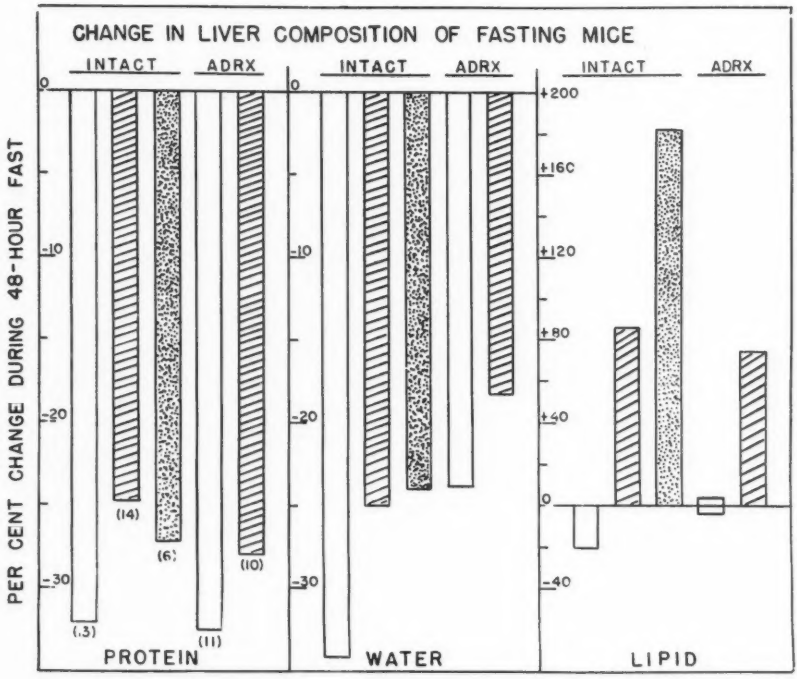

FIG. 2. The length of the bars represents per cent change in liver constituents of fasted mice related to the tissue composition of fed controls taken as a zero baseline. Comparison is made between intact fasted *vs.* intact fed control, and adrenalectomized fasted, *vs.* adrenalectomized fed, animals. (*v.*, Results, section 1.). The absolute amounts of each constituent present at the start of the fast are inferred for each group from the liver weight: body weight ratio and liver composition found by simultaneous analysis of fed controls. The open bars represent untreated, fasted animals, the cross-hatched bars, animals receiving 200 micrograms of growth hormone, and the stippled bars, those to which the 4 mgm. dose of hormone was administered. The first three bars in each section refer to intact animals, the last group in each section represents adrenalectomized mice. The number of animals contributing to the average in each case is indicated by the number enclosed in parentheses. Negative values indicate losses, positive values, gains.

that used for protein and water in order to show the intense lipid infiltration produced; the 4 mgm. dose increased the total lipid by almost 200%. It is possible that these values were observed at a time beyond the maximum lipid accumulation (cf. Steppuhn, 1934; Campbell, 1938; and Hodge et al., 1948). Even in fasting, adrenalectomized animals which, in confirmation of MacKay and Barnes (1937a) were unable to mobilize lipid to the liver, the administration of growth hormone produced a similar increase in liver lipid.

Table 2 demonstrates the influence of growth hormone on liver weight of fasting mice. It will be observed that liver wet weight per 100 gm. final body weight was significantly greater in growth hormone-treated animals, both intact and adrenalectomized, than in their respective fasting controls, despite the fact that the adrenalectomized mice began the fast at a lower initial level. In addition, fat-free liver weight, and total dry, fat-free weight were with one exception significantly greater in growth hormone-treated, fasted mice than in their fasted controls, demonstrating again the relative retention of nitrogen even when computed on a fat-free, and on a water-free basis. Thus, notwithstanding the fact that nitrogen *concentration* was reduced because of the tremendous lipid infiltration, the relatively greater liver mass in the growth hormone-treated animals prevented the nitrogen from reaching as low levels as those seen in fasting alone. This is depicted in Table 3, which shows the greater amounts of protein and water *as well as* lipid in the livers of growth

TABLE 3. THE INFLUENCE OF GROWTH HORMONE ON LIVER COMPOSITION IN MALE CBA MICE DURING FASTING†

Group	No. of Animals	Liver Nitrogen, %	Total Liver Protein (gm.)	Liver Water, %	Total Liver Water, (gm.)	Liver Lipid, %	Total Liver Lipid, (gm.)
Intact							
Fed	8	3.48 ± 0.045	0.296 ± 0.010	69.7 ± 0.39	0.955 ± 0.045	7.52 ± 0.27	0.103 ± 0.006
Fasted 48 hrs.	13	3.64 0.045 ±	0.196 ± 0.007	68.3 ± 0.59	0.604 ± 0.030	8.85 ± 0.64	0.079 ± 0.008
Fasted 48 hrs.+ Growth Hormone, 200 μgm.	14	3.16 ± 0.078*	0.218 ± 0.006*	63.0 ± 1.18*	0.697 ± 0.020*	16.4 ± 1.26*	0.187 ± 0.020*
Fasted 48 hrs.+ Growth Hormone, 4 mgm.	6	2.77 ± 0.18*	0.212 ± 0.005	58.0 ± 2.19*	0.712 ± 0.020*	23.0 ± 3.0*	0.295 ± 0.056*
Adrenalectomized							
Fed	13	3.61 ± 0.062	0.252 ± 0.007	70.6 ± 0.40	0.792 ± 0.030	7.51 ± 0.32	0.083 ± 0.003
Fasted 48 hrs. (+ saline)	11	3.26 ± 0.08	0.181 ± 0.006	71.5 ± 0.70	0.636 ± 0.016	9.78 ± 0.82	0.088 ± 0.010
Fasted 48 hrs.+ Growth Hormone 200 μgm.	10	2.99 ± 0.07*	0.192 ± 0.005	66.0 ± 1.55*	0.679 ± 0.021*	15.21 ± 1.85*	0.157 ± 0.019*

† Deviations shown are standard errors.
* The starred values are significantly different from the respective fasted control figures.

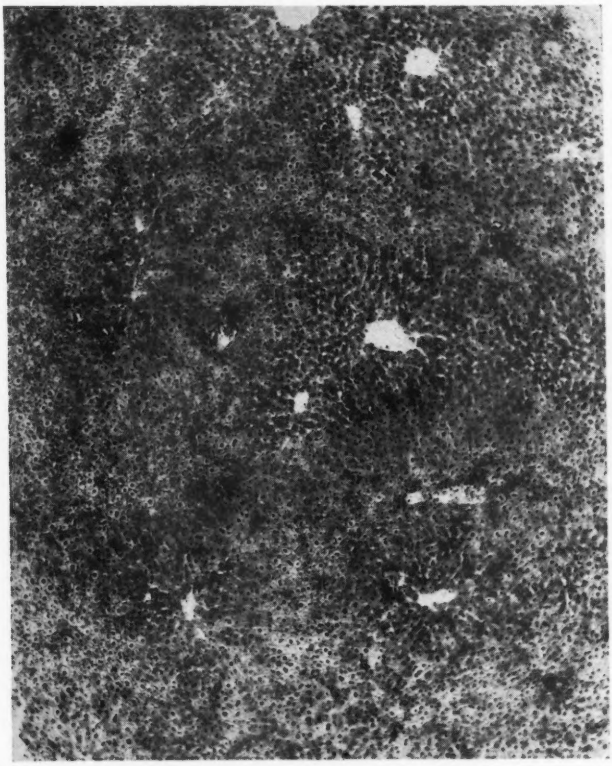

FIG. 3. Photomicrograph of a section of the liver of a mouse which had received 200 micrograms of growth hormone during a 48-hour fast. Hematoxylin-eosin stain; magnification ×65; lipid content, 16.8% of the fresh weight. Note preservation of architecture, light-staining (vacuolated) periportal areas, considerable variation in nuclear size, and absence of necrosis.

hormone-treated animals compared with fasting controls. These data are statistically significant where indicated.

b. *Histological appearance.*[4]

In spite of intense hepatic lipid infiltration, there was no microscopic evidence of any essential disturbance of lobular architecture in any of the series of livers from fasting animals treated with growth hormone. No sign of necrosis was observed even with lipid concentrations up to 40% of the wet weight of the liver, although some variation in nuclear size has been noted. A typical photomicrograph is reproduced in Fig. 3. Sudan IV and Sudan Black stains indicated a periportal lipid distribution, with sparing of the central areas in most

[4] Dr. Henry Bunting of the Department of Pathology and Dr. Elijah Adams of the Department of Physiological Chemistry, Yale University, kindly aided in the interpretation of histological sections of liver and other tissues.

cases. The intensity of Sudan staining was well correlated with lipid content found by chemical analysis and progressive increases in staining capacity were observed: from slight, seen in livers of control fasted series (8–10% total lipid), through moderate, with the 200 microgram dose of growth hormone (15–16% lipid), to intense, resulting from the administration of 4 mgm. growth hormone during the fast (average 23% lipid). Livers from fed animals, with an average lipid content of 7.5% of the wet weight of the liver, showed no Sudan staining. The above remarks on liver lipid distribution under the influence of growth hormone in fasting mice also apply in general to the adrenalectomized group, in which, however, sparing of central areas was less pronounced. Preliminary evidence obtained from fractionation studies indicated that the bulk of the excess liver lipid accumulated by growth hormone-treated, fasted animals was neutral fat.

3. *The influence of growth hormone on the composition of weight loss of fasting mice.*

Figure 4 demonstrates the effect of growth hormone on the composition of weight loss of the residual carcass of growth hormone-treated *vs.* control fasted mice. These data are plotted in terms of grams of each constituent lost per 100 gm. initial body weight. It will be observed from the figure and also from Table 4 that a distinct and significant reduction in body weight loss due to fasting was brought about by both doses of growth hormone in intact animals

TABLE 4. THE INFLUENCE OF GROWTH HORMONE ON THE COMPOSITION OF THE WEIGHT LOSS OF FASTING CBA MICE

Group	No. of Mice	Orig. B.W. (gm.)	Wt. Loss (gm.)[1]	"p" Value[2]	Ave. Final N (%)[3]	Wt. Loss as Prot. (gm.)[4]	Ave. Final H_2O (%)[3]	Wt. Loss as H_2O (gm.)[4]	Ave. Final Lipid (%)[3]	Wt. Loss as Lipid (gm.)[4]	Ratio Prot.: H_2O Loss	Ratio Prot.: Lipid Loss
Intact												
Fed	8	22.4	—	—	3.15	—	66.3	—	9.95	—	—	—
Fasted 48 hrs.	13	21.8	5.5± 0.26	—	3.46	1.13	68.5	3.71	4.74	0.40	1:3.26	1:0.354
Fasted+Growth Hormone 200 μgm	14	22.0	4.6 ± 0.17	<0.01	3.45	0.95	69.0	3.12	5.09	0.35	1:3.30	1:0.369
Fasted+Growth Hormone, 4 mgm.	6	22.2	4.2 ± 0.26	<0.01	3.28	0.85	68.1	2.82	4.98	0.32	1:3.34	1:0.376
Adrenalectomized												
Fed	9	20.6	—	—	3.16	—	67.6	—	—	—	—	—
Fasted 48 hrs. (+ saline)	13	21.8	2.4 ± 0.23	—	3.16	0.48	71.3	1.67	—	—	1:3.50	—
Fasted 48 hrs +Growth Hormone 200 μgm.	9	21.7	2.0 ± 0.27	<0.2	3.23	0.41	71.6	1.39	—	—	1:3.42	—

[1] Standard errors are shown.

[2] Between each group given growth hormone and the fasted control.

[3] All analyses refer to whole carcass minus liver, gut and a constant proportion of lymphoid tissue.

[4] The contribution of each constituent to the weight loss is calculated by multiplying the average composition between initial and final levels by the observed weight loss.

("p" value <0.01). This was due, in the main, to reduced loss of protein and water. The differences in carcass lipid loss between control and growth hormone-treated animals are probably not significant because of the greater variability of initial carcass fat depots. However, Table 4 reveals that a somewhat greater amount of carcass lipid is lost per unit of protein loss in the growth hormone-treated animals.

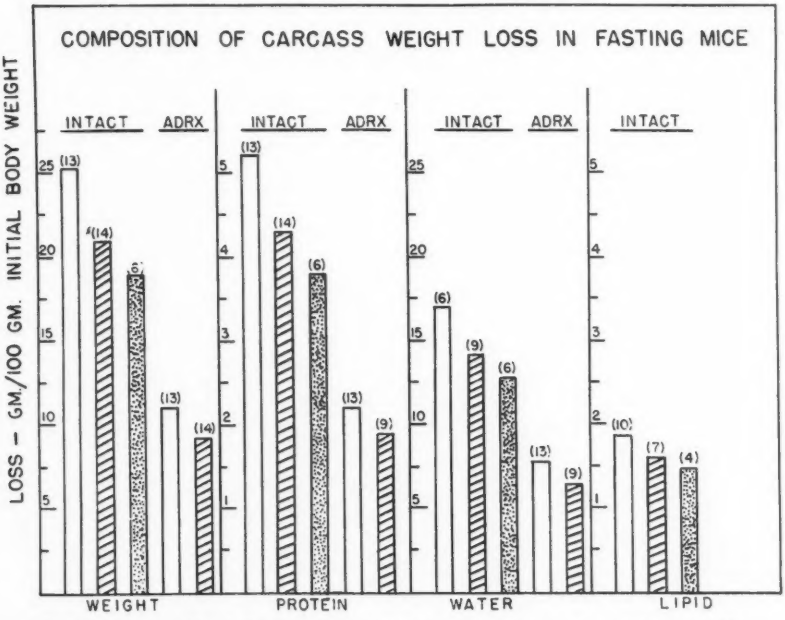

FIG. 4. The length of the bars represents loss of each constituent in gm./100 gm. initial body weight. The contribution of each constituent to the weight loss is calculated from the average composition during the fasting period, between initial (appropriate control) and final (observed) levels, and multiplying by observed weight loss. The open bars represent untreated, fasted animals, the cross-hatched bars, animals receiving 200 micrograms of growth hormone, and the stippled bars, those to which the 4 mgm. dose of hormone was administered. The first three bars in each section refer to intact animals, the last group in each section represents adrenalectomized mice. The number of animals contributing to the average in each case is indicated by the number enclosed in parentheses.

In adrenalectomized animals treated with growth hormone, there appeared to be a relative retention of nitrogen and water in the carcass, although the magnitude of the changes is not statistically significant (Table 4, Figure 4).

4. *The composition of lymphoid tissue weight change in fasting mice.*

In Figure 5 and Table 5 are indicated the fasting changes in lymphoid tissue composition. It will be observed that a profound fasting

involution of lymphoid tissue occurred in intact, untreated animals (cf. White and Dougherty, 1947). This was augmented, probably as a result of non-specific stress, by the large dose of growth hormone (4 mgm.), but this augmentation was of limited significance ($p = 0.1$). The lower dose of growth hormone was subsequently employed because, although it did not accelerate the fasting involution of lymphoid

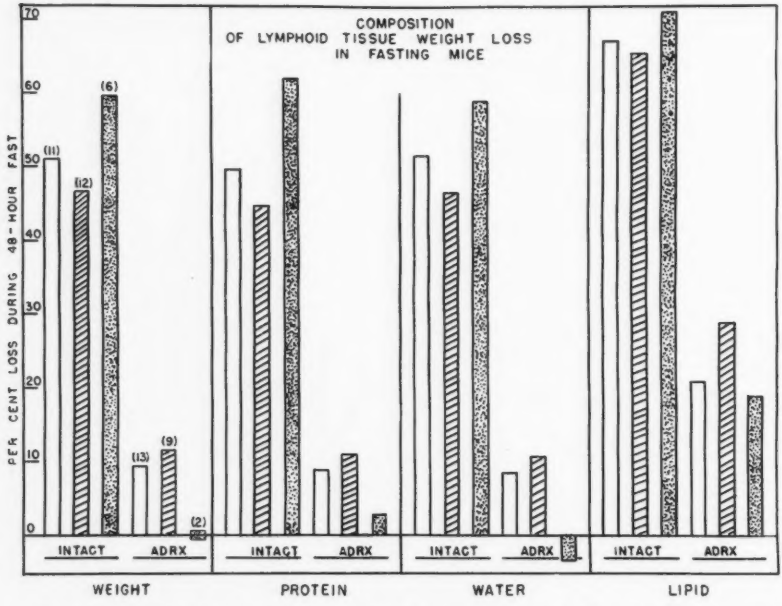

FIG. 5. The length of the bars represents per cent loss in lymphoid tissue constituents of fasted mice related to the tissue composition of fed controls taken as a zero baseline. Comparison is made between intact fasted *vs.* intact fed control, and adrenalectomized fasted *vs.* adrenalectomized fed, animals. (*v.*, Results, section 1.). The absolute amounts of each constituent present at the start of the fast are inferred for each group from the lymphoid tissue weight: body weight ratio and composition found by simultaneous analysis of fed controls. The open bars represent untreated, fasted animals, the cross-hatched bars, animals receiving 200 micrograms of growth hormone, and the stippled bars, those to which the 4 mgm. dose of hormone was administered. The first three bars in each section refer to intact, the second three to adrenalectomized, animals. The number of animals contributing to the average in each case is indicated by the number enclosed in parentheses. Values falling on the baseline indicate no change from, and values below the baseline, gain over, the respective control level.

tissue in the intact animal, it was nevertheless active in producing other effects (cf., sections 2, 3).

Protein and water were lost from lymphoid tissue in similar proportions during fasting, regardless of growth hormone administration, but lipid appeared to be further reduced, relative to total lymphoid tissue loss. This may indicate a selective mobilization of lipid beyond that lost as a consequence of dissolution of individual cells.

TABLE 5. THE COMPOSITION OF LYMPHOID TISSUE IN FASTING MICE±

Group	No. of Mice	Wet wt. Lymphoid Tissue (gm./100 gm. B.W.)	Loss in Total Lymphoid Tissue Wt. (%)	Percentage Composition		
				Nitrogen	Water	Lipid
Intact						
Fed	8	0.870 ± 0.039	—	2.65 ± 0.05	77.7 ± 0.6	7.69
Fasted 48 hrs.	11	0.555 ± 0.042	51.0	2.75 ± 0.04	78.2 ± 1.0	5.20
Fasted 48 hrs.+ 200 μgm. Growth Hormone	12	0.587 ± 0.023	46.6	2.75 ± 0.03	77.9 ± 0.2	4.9†
Fasted 48 hrs.+ 4 mgm. Growth Hormone	6	0.444 ± 0.040[1]	59.5	2.43 ± 0.09*	77.0 ± 0.5	5.37
Adrenalectomized						
Fed	9	1.179 ± 0.088	—	2.64 ± 0.02	78.9 ± 0.3	4.6†
Fasted 48 hrs. (+saline)	13	1.202 ± 0.065	9.3	2.65 ± 0.04	79.4 ± 0.2	4.0†
Fasted 48 hrs.+ 200 μgm. Growth Hormone	9	1.148 ± 0.064	11.7	2.65 ± 0.03	79.7 ± 0.2	3.7†
Fasted 48 hrs.+ 4 mgm. Growth Hormone	2	1.225	0	2.52	80.5	3.7†

± Deviations shown are standard errors.
† By difference.
* The starred values are significantly different from the respective fasted control figures.
[1] Of limited significance: $p = 0.1$.

This disproportionately greater loss of lymphoid tissue lipid, compared to losses in other constitutents, appeared to occur also in adrenalectomized animals during fasting, even though there was no significant loss in nitrogen or water. Actually, the total lymphoid tissue weights, per 100 gm. body weight, were not significantly affected by fasting, or by either of the two growth hormone doses (Table 5.) in the adrenalectomized group. When, on the other hand, lymphoid tissue of fasting adrenalectomized mice is compared to the corresponding structures of fasting intact animals, ample evidence of growth after adrenalectomy is observed from these data.

DISCUSSION

The catabolic influence of the adrenal cortical steroids, as evidenced by their capacity to accelerate the rate of nitrogen excretion (cf. review by White, 1948), is generally acknowledged. In the adrenalectomized, fasting rat, urinary nitrogen falls to a low level, suggesting a retardation of the rate of tissue catabolism (Evans, 1936; Harrison and Long, 1940). This is further exemplified in the reduction in nitrogen loss from carcass and lymphoid tissue reported by White and Dougherty (1947) in fasting, adrenalectomized mice. The results re-

ported in the present investigation indicate that even this low rate of tissue breakdown can be further suppressed or counteracted by the administration of growth hormone (or of anterior pituitary extracts possessing growth-promoting activity, cf., Harrison and Long, 1940), and constitute additional evidence of the opposing actions of growth and adrenal cortical hormones (cf., Marx et al., 1943). In the present investigation, growth hormone exerted its nitrogen-sparing effect in the adrenalectomized fasting mouse primarily in the liver.

The administration of growth hormone to the fasting mouse resulted in a diminished rate of nitrogen and water loss, associated with an apparent acceleration of fat metabolism. These observations are analagous to the effects noted in the fed animal by Lee and Schaffer (1934). The weight gain of fed rats treated with semi-purified growth-promoting extracts was composed of an increased proportion of nitrogen, water, and ash, and a decreased concentration of fat, as compared to control, pair-fed litter mates. It was deduced from the latter studies that chemical maturation, according to the concepts of Moulton (1923), was delayed by treatment of the animals with anterior pituitary extract.

The shift to an increased rate of fat oxidation, inferred from the earlier work with crude anterior pituitary extracts (cf., Schäfer (1931), Wadehn (1932), Gaebler (1933), Lee and Schaffer (1934), Steppuhn (1934), Best and Campbell (1936), Harrison and Long (1940), and others), was reflected in the present studies primarily by the metabolic events occurring in the liver. The slight hepatic fatty infiltration which has been observed as a result of fasting alone, was strikingly exaggerated by the administration of growth hormone to the intact animal. This treatment resulted in exceedingly fatty livers, which were, however, histologically free from necrosis or inflammatory processes. Downs (1930) and Best and Campbell (1936) described liver necrosis following injection of crude anterior pituitary extracts; the latter authors also observed fatty infiltration of the liver. Purified growth hormone has also been reported recently by Bennett et al. (1948) to possess ketogenic properties. Ketosis was prevented by removal of the liver in the latter studies, as well as in the earlier experiments of Mirsky (1936) who used crude extracts. It was further observed by Shipley (1944) that ketogenesis by rat liver *in vitro* was accentuated by anterior pituitary extract. These studies indicated the importance of the liver in mediating the accelerated fat metabolism. It is possible that the liver may be essential for other manifestations of growth hormone activity. Thus, Frame and Russell (1946) found that whole anterior pituitary extract, presumably rich in growth-promoting activity, decreased the blood amino acid content of normal rats, but was without effect in the eviscerated animal. The liver has also been directly implicated in the earliest observed effects of anterior pituitary extracts i.e., reductions in liver non-protein

nitrogen and urea (Lee and Schaffer, 1934), and in glutathione (Gregory and Goss, 1939). It seems probable from these data that the liver may be a principal site of action of growth hormone (cf., Lee, 1936).

The present studies reveal that purified growth hormone is capable of causing an increase in liver fat in the fasting, adrenalectomized animal notwithstanding the impairment of fasting lipid mobilization to the liver in the absence of the adrenals (cf., also MacKay and Barnes, 1937a). It had been shown by Fry (1937) and MacKay and Barnes (1937a) that crude anterior pituitary extracts failed to induce fatty liver in the adrenalectomized rat. Adrenalectomy also prevented the fatty infiltration of the liver which usually accompanies pancreatectomy (Long and Lukens, 1936), diminished the deposition of liver fat following partial hepatectomy (MacKay and Carne, 1938; Berman et al., 1947), and reduced the ketonuria of fasting rats after anterior pituitary extract administration (Fry, 1936; MacKay and Barnes, 1937b). It is apparent, however, that under the influence of the more highly purified growth hormone fraction used in the present investigation, fat mobilization to the liver was independent of the adrenal. The ketogenic effects of purified growth hormone have also been observed in the absence of the adrenal by Bennett et al., (1948). The available evidence suggests that the shift to accelerated fat metabolism promoted by growth hormone is not necessarily adrenal-mediated. The interpretation of the earlier data demonstrating that the presence of the adrenal is essential for liver lipid accumulation with crude anterior pituitary extracts is made difficult by the complex nature of the extracts used.

In view of the profound effects of growth hormone on the metabolic pattern of the fasting animal, it is somewhat surprising that lymphoid tissue appeared to be independent of its influence. Neither the composition nor the weight of the lymphoid structures of the intact, fasted animal was altered by growth hormone administration. The accentuated involution of lymphoid tissue produced by very large doses of this hormone was probably due to a non-specific augmentation of pituitary-adrenal cortical secretion. Marx, Simpson, Reinhardt and Evans (1942), however, reported some increase in thymus weight beyond that expected as a result of body weight gain in fed rats injected chronically with relatively pure growth hormone.

The increased mass of lymphoid structures seen following adrenalectomy appeared to fulfill the biochemical criteria of true growth i.e., the deposition of relatively larger amounts of nitrogen and water and a lower proportion of fat as compared to the adult structures. This growth occurs notwithstanding the obvious drain on the energetic resources of the animal during fasting, and is further evidence of the decreased rate of utilization of lymphoid tissue nitrogen in the ab-

sence of the adrenal steroids (White and Dougherty, 1947). The data appear to suggest, however, that the lipid of lymphoid tissue is a more labile component, and may be available to the fasting animal even in the absence of the adrenals. The growth of lymphoid tissue following adrenalectomy could not be potentiated by growth hormone in the fasting animal. Thus, the rôle of the adrenal in regulating the structure and function of lymphoid tissue gains emphasis from the failure to influence this tissue with growth hormone, which, in general, has a nitrogen-sparing effect in the fasting animal.

The metabolic effects of anterior pituitary extracts have been reviewed by Houssay (1942) and Long (1942, 1943), and, more recently, for growth-promoting fractions of a greater degree of purity, by Marx and Evans (1944) and Li and Evans (1947). There appear to be several possible mechanisms by which growth hormone may act to produce the metabolic changes observed in the present investigation and in other studies. The evidence is consistent with the alternatives a), that growth hormone inhibits the rate at which the animal is able to mobilize or utilize protein and is therefore compelled to burn a greater proportion of fat to supply its energy needs, or, b), that growth hormone accelerates the rate at which the animal can mobilize fat—i.e., that it burns fat preferentially, and consequently less protein is required as an energy source. A fat mobilizing factor in the cruder pituitary extracts was termed "lipoitrin" by Raab (1934). The magnitude and early onset (Campbell, 1938) of fat mobilization following anterior pituitary extract injections suggest that the effects of growth hormone on the nitrogenous constituents of liver and other tissues may be secondary. The influence of anterior pituitary extract in promoting fat metabolism has been advanced by others (Gaebler, 1933; Lee and Schaffer, 1934; and Long, 1943) as a possible explanation for the mechanism by which protein could be spared. Growth hormone may produce at least a portion of its effects by reducing total metabolism (Kleiber and Cole, 1939), although this was not seen in earlier experiments by Gaebler (1933, 1935), who reported a large calorigenic response concomitant with a fall in R.Q. in dogs treated with crude anterior pituitary extracts. Whatever the primary mechanism of growth hormone action may be, it appears to be mediated by, or require the participation of, the liver.

In considering the economy of the fasting animal, it is possible that growth hormone production is stimulated by fasting, as appears to be the case for adrenotrophin. This suggestion is supoprted by the observation that the untreated, fasting animal burns a larger proportion of lipid than does the fed subject. The latent period during which the urinary nitrogen falls progressively to a minimal level as fasting proceeds may be due to the time required for the activity of endogenous growth hormone to be manifested, and for adrenal exhaustion and consequent decline of the catabolic effects of the adrenal cortical steroids.

SUMMARY

The influence of a purified growth hormone preparation on fasting metabolism of intact and adrenalectomized mice has been investigated. Doses of 200 micrograms, administered during a 48-hour fasting period, caused a depression of the rate of nitrogen and water loss from liver and carcass of these animals. This relative sparing of nitrogen was accompanied by an acceleration of fat metabolism as evidenced by intense fatty infiltration of the livers of fasting animals treated with growth hormone. The accumulation of liver lipid as a result of growth hormone injection was independent of the presence of the adrenals. Histological examination did not reveal evidence of liver damage. The data support the suggestion that the lipid mobilizing action of growth hormone may be integrated with its nitrogen sparing effect.

The involution of lymphoid tissue in the fasting mouse was independent of the influence of growth hormone, except at very large doses (4 mgm./48 hours), which accentuated this phenomenon in intact mice. The increase in mass of lymphoid tissue following adrenalectomy was not potentiated by growth hormone administration during fasting. Analysis of the composition of the lymphoid tissue weight gain following adrenalectomy yielded evidence that this enlargement conformed to the criteria of true growth.

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Miss Jeanette Opsahl and Miss Rita Quan contributed valuable assistance in the analytical procedures.

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SEASONAL CHANGES IN THE TESTES, EPIDIDYMIDES AND SEMINAL VESICLES OF DEER INVESTIGATED BY HISTOCHEMICAL METHODS[1]

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IT WAS shown some years ago that the testes, epididymides and seminal vesicles of Virginia deer exhibit seasonal changes in respect to their weights and histological appearances (Wislocki, '43). In the latitude of New England, they increase in weight and reach maximal activity in the fall (October, November), followed by a marked decline in weight and suppression of secretory activity and spermatogenesis in the spring (May, June).

Since the former report was published, the internal sex organs of male deer have been reinvestigated in this laboratory by a variety of histochemical methods for lipids (including steroids), nucleoproteins, acid mucopolysaccharides, glycogen and phosphatases. The present paper describes these reactions in the testes, epididymides and seminal vesicles of Virginia and Japanese deer, comparing the results in the active and inactive states.

MATERIALS AND METHOD

The material consists of the testes and seminal vesicles of 4 Virginia deer (Odocoileus virginianus borealis) and 2 Japanese or Sika deer (Cervus nippon).[2] The 4 Virginia deer, from a healthy herd on free range, were obtained on June 9 and 14, October 19 and November 30. The 2 Japanese deer, from a small captive herd, were taken on June 13 and November 13.

For the staining of lipids, pieces of tissue were fixed in 10% neutral formalin. Frozen sections were stained in either sudan III (sat. sol. in 70% alcohol with 1% pot. hydrox. added) or sudan black B (sat. sol. in 70% alcohol) for 7 minutes and then mounted in glycerine jelly.

For the demonstration of steroid substances, pieces of testes were fixed in 10% neutral formalin. Unstained, frozen sections of these blocks were examined in ultraviolet light for natural fluorescence and with the polarizing

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microscope for birefringence, while other sections were stained with sudan III or sudan black B and with Schiff's plasmal reagent (the plasmal reaction of Feulgen and Voit). Other sections were extracted with acetone at room temperature before carrying out the above procedures.

No single method is available for identifying ketosteroids in tissue sections, but by the use of a combination of methods, the sum of which characterizes ketosteroids (but no other known type of compound) they can be identified with some assurrance (Bennett, '40; Dempsey and Wislocki, '46). Since steroids are soluble in acetone at room temperature, the other methods used will be negative following extraction of the sections in that solvent. The steroids, along with other lipids, are sudanophilic and hence will be stained with sudan dyes. Because of their ketone groups, the ketosteroids will recolorize leucofuchsin (Schiff's reagent) to produce a purple color (the "plasmal reaction" of Feulgen and Voit). Unsaturated steroids exhibit greenish or yellowish autofluorescence when illuminated by ultraviolet light, and are visible in cells as birefringent spherocrystals.

For the investigation of cytoplasmic basophilia, blocks of tissue were fixed in Zenker's fluid and the deparaffinized sections were stained with eosin and methylene blue or with toluidin blue (½% aqueous sol.). The Feulgen reaction for desoxyribonucleic acid was carried out by the standard technique after fixation in Zenker's fluid.

Acid mucopolysaccharides were sought for by means of their metachromatic staining. Pieces of tissue were fixed in 4% basic lead acetate and deparaffinized sections were stained in a ½% aqueous solution of toluidin blue for 30 minutes (Holmgren, '40; Wislocki, Bunting and Dempsey, '47).

Glycogen was sought for in pieces of tissue fixed in Rossman's fluid (abs. alc. sat. with picric acid, 90 cc.; formaldehyde (40%), 10 cc.). Deparaffinized sections were stained by both the Bauer-Feulgen and periodic acid-Schiff methods. Control sections were exposed to saliva for 30 minutes at room temperature before staining. The periodic acid-Schiff method was applied according to the directions of McManus ('46). The deparaffinized sections are placed in a 1% solution of periodic acid for 5 minutes; they are then washed and stained for 10 to 15 minutes in Schiff's reagent, transferred directly to 2 changes of sulphurous acid for 5 minutes each, then cleared and mounted in balsam.

Besides glycogen, the periodic acid-Schiff method stains other substances and structures, including mucus, cartilage matrix, collagenous fibers, basement membranes, fibrin and the elastica interna of blood vessels (Lillie et al., '47; Wislocki and Dempsey, '48). Besides glycogen, the Bauer-Feulgen reaction which employs chromic acid in a way similar to periodic acid stains mucus, cartilage matrix and elastica interna (Wislocki, Bunting and Dempsey, '47). Unlike the staining of glycogen, these latter reactions are not prevented by previous treatment of the sections with saliva. Staining by these 2 methods depends probably on the oxidation of polysaccharide complexes in various glycoproteins by periodic or chromic acid, with the result that aldehydes are formed. The latter are revealed by their reaction with the leucofuchsin in Schiff's reagent.

Alkaline phosphatase was investigated by the method of Gomori ('41a) as modified by Dempsey and Deane ('46) utilizing sodium glycerophosphate, fructose diphosphate and yeast nucleic acid as substrates. Pieces of tissue

were fixed in chilled 80% alcohol and the sections incubated at pH 9.4 for 3 and 6 hours. The sections were counterstained with Mayer's paracarmine.

Acid phosphatase was demonstrated by Gomori's method ('41b) in tissues fixed in chilled acetone and incubated in glycerophosphate at pH 4.7 for 24 and 48 hours. The sections were counterstained with Mayer's paracarmine.

Besides the use of these more specific histochemical methods occasional sections were stained by other techniques. The latter included staining by Masson's triacid mixture (ponceau-acid fuchsin, orange G and light green), hematoxylin and eosin, Heidenhain's iron-hematoxylin method, Bodian's protargol methods as modified by Dawson and Barnett ('44) and Pap's silver impregnation method for reticulum (Pap, '29; Mitchell and Wislocki, '44).

TESTIS

The testes of deer killed in New England are active and large at the time of the rutting season in the fall and are small and atrophic in the spring (Wislocki, '43). The present investigation compares the cytology and histochemistry of the testes of several Virginia and Japanese deer at these 2 periods when the seasonal contrasts are maximal. The intermediate phases of the intervening months have not been followed.

THE INTERSTITIAL TISSUE

The interstitial tissue of the testes is more voluminous in the rutting season than in the sexually quiescent months of the spring. In the fall the cells are large and numerous and their cytoplasm is abundantly filled with sudanophilic lipid droplets (figs. 1 and 10). Sections stained by Schiff's reagent reveal an intense plasmal reaction (figs. 4 and 11) which appears to coincide with the sudanophil droplets (cf. figs. 10 and 11). Observed under the polarizing microscope, many birefringent droplets are encountered and when examined for fluorescence in ultraviolet light the interstitial cells emit a yellowish autofluorescence. These reactions are lost after preliminary extraction of the frozen sections with acetone.

In June testes the cells are smaller and fewer in number and the sudanophilic droplets are diminished (fig. 12). The Schiff reaction, birefringence and autofluorescence are correspondingly reduced. The above observations indicate not only that the interstitial tissue is the site of formation of ketosteroids but that the quantity present is much greater in the fall at the time of rut than during the period of sexual inactivity.

In the course of preparation of paraffin sections of the testes the fat droplets are ordinarily dissolved out, giving a delicate foamy appearance to the cytoplasm of the interstitial cells. This is apparent in sections fixed in Zenker's fluid and subsequently stained with eosin and methylene blue or with Masson's triacid mixture.

In the fall testes, fixed in Zenker's fluid and stained with eosin and methylene blue, the cytoplasm of the interstitial cells stains a

lavender color with superimposed delicate basophilic stippling. In June the shrunken cytoplasm of the interstitial cells is paler in color and shows practically no cytoplasmic basophilia.

With toluidin blue, after fixation in 4% basic lead acetate, metachromasia has not been encountered in the interstitial tissue at either time of the year, an observation indicating the absence of any acid mucopolysaccharides.

Crystals have not been encountered in the interstitial cells of deer, although they were sought for in sections stained with Heidenhain's iron-hematoxylin, eosin and methylene blue and Masson's triacid stain.

By the Bauer-Feulgen and periodic acid-Schiff procedures glycogen is not demonstrable in the interstitial cells, but it occurs with regularity in the walls of the arteries and arterioles. By the periodic acid-Schiff method the basement membranes surrounding the seminiferous tubules and collagen in general are intensely colored (fig. 3), but, since this staining is not prevented by preliminary exposure of the sections to saliva, it is not due to glycogen. Similarly, in June testes glycogen is present in the walls of the blood vessels but is absent from the interstitial cells. With the periodic acid-Schiff method the fibrous stroma of June testes is especially deeply stained, a result attributable to the relative increase in density of the collagen in the shrunken organ.

The cytoplasm of the interstitial cells in the fall testes contains little alkaline phosphatase, whereas the basement membranes enclosing the seminiferous tubules and the walls of the interstitial arteries and arterioles are intensely stained (figs. 5 and 6). With fructose diphosphate as substrate the cytoplasm of the interstitial cells is more distinctly stained (fig. 5) than after the use of yeast nucleic acid (fig. 6). In the interstitial tissue of June testes, on the contrary,

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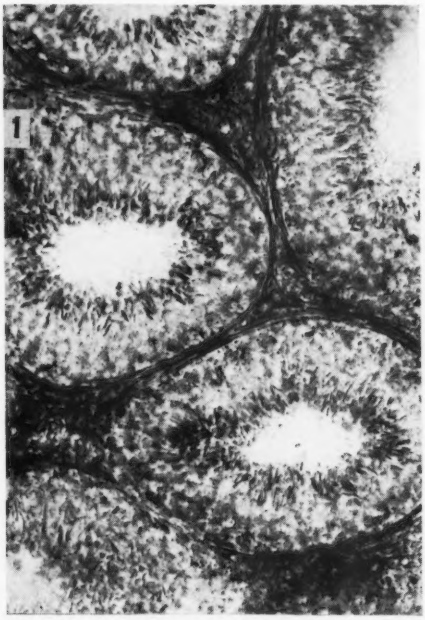
Explanation of Figures

1. Frozen section of a formalin-fixed testis of a Virginia deer (Nov. 30) stained with sudan III for 7 minutes. Observe the staining of the interstitial tissue and the seminiferous tubules. ×160.

2. Frozen section of formalin-fixed testis of a Japanese deer (Nov. 13) stained with sudan black B for 7 minutes. Notice the stained lipid droplets in the elements of the seminiferous tubule and compare with figure 1. The sperm heads, seen in profile, are accentuated by sudan dyes. ×70 ocular, ×90 objective.

3. Section of a testis of a Virginia deer (Oct. 19) fixed in Rossman's fluid and stained by McManus' periodic acid-Schiff procedure. Observe the staining of the basement membranes enclosing the seminiferous tubules and of the walls of the blood vessels of the interstitial tissue. The interstitial cells are unstained. Notice the relatively faintly stained spermatids in the 2 tubules in the lower half of the photograph and compare them with the cells shown in figure 15. In the tubule visible in the upper half of the photograph, observe the more deeply stained spermatozoa and compare them with figure 16. ×260.

4. Frozen section of a formalin-fixed testis of a Virginia deer (Nov. 30) stained with Schiff's reagent illustrating the prominent plasmal reaction of the interstitial tissue. Compare this photograph with a drawing of the interstitial tissue at higher magnification (fig. 11). ×160.



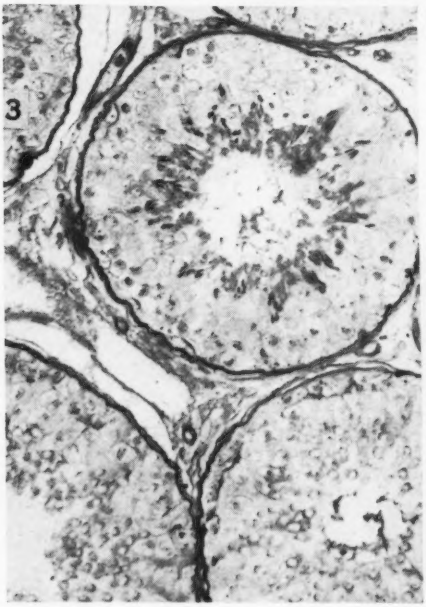


PLATE 1

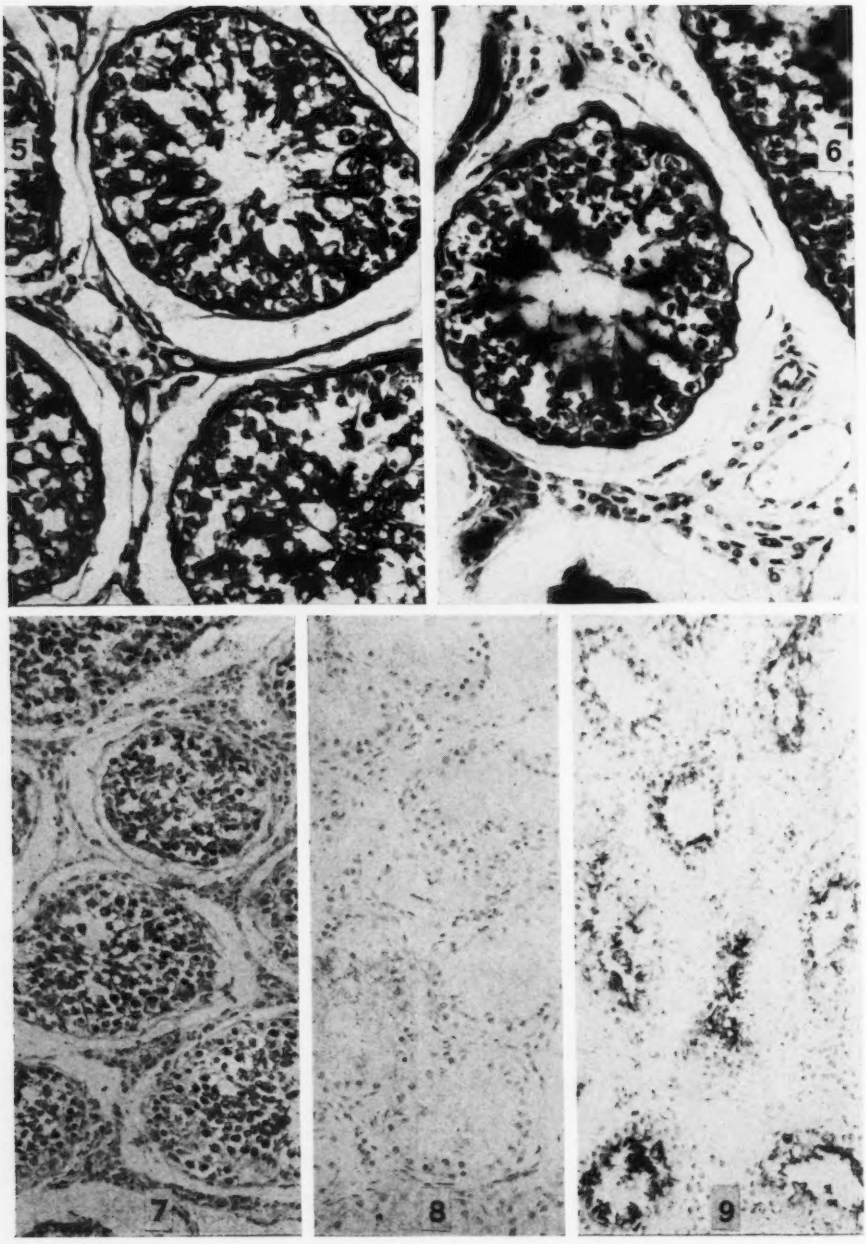


PLATE 2

alkaline phosphatase is practically absent (fig. 9). It should be noted that the staining of the nuclei of the interstitial cells registered in photographs 5, 6 and 9 is due mainly to counterstaining by paracarmine and not to the presence of alkaline phosphatase.

Acid phosphatase is present in slight amounts in the interstitial tissue of fall testes (fig. 7). Differing from alkaline phosphatase, it is present principally in the cell nuclei and does not occur in either the arterial walls or in the basement membranes. In June testes it is absent (fig. 8).

SEMINIFEROUS TUBULES

Figures 1 and 2 illustrate frozen sections of fall testes stained respectively with sudan III and sudan black B. Both coarse and fine lipid droplets occur within the seminiferous tubules. The coarse particles vary in size and are located irregularly at the periphery of the tubules near the basement membrane. The minute lipid particles are widely scattered, existing both at the periphery and toward the center of the tubules. In the latter region they are maximal in number and appear to be located within the cytoplasm of spermatids as well as within globoid cytoplasmic masses freed into the lumen of the tubules during cytomorphosis of spermatids into spermatozoa. In addition to these various lipid droplets, the heads of the spermatozoa appear to be faintly stained, especially when viewed in profile (fig. 2). Since the large sudanophil droplets near the basement membrane do not seem

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Explanation of Figures

All of the sections illustrated on this plate were counterstained with Mayer's paracarmine.

5. Section of testis of a Virginia deer (Oct. 19) showing the distribution of alkaline phosphatase. Fixed in chilled 80% alcohol. The section was incubated in fructose diphosphate at pH 9.4 for 6 hours. ×220.

6. The same, utilizing yeast nucleic acid as substrate and incubating at pH 9.4 for 6 hours. In this and the previous photographs observe the intense phosphatase reaction of the seminiferous tubules and the surrounding basement membranes. The interstitial tissue is quite faintly stained excepting the dark reaction of the walls of the arteries. The apparent nuclear staining in this and the preceding section is partly due to the red color imparted by the counterstain. ×220.

7. Section of a testis of a Japanese deer (Nov. 13) showing the pattern and intensity of acid phosphatase. Fixation in chilled acetone. Section incubated with sodium glycerophosphate at pH 4.7 for 48 hours. The reaction is confined mainly to the cells of the seminiferous tubules. ×140.

8. Section of a testis of a Japanese deer (June 13) incubated with sodium glycerophosphate at pH 4.7 for 48 hours. Observe the difference between the November testis (preceding photograph) and the June testis shown in this figure. In June the shrunken, inactive seminiferous tubules, as well as the interstitial tissue, are essentially negative for acid phosphatase. ×140.

9. Section of a testis of a Japanese deer (June 13) incubated with sodium glycerophosphate at pH 9.4 for 6 hours. Fixation in chilled 80% alcohol. The relatively slight reaction for alkaline phosphatase is confined almost entirely to the contents of the shrunken tubules. Neither the basement membranes nor the blood vessel walls have reacted. Compare with the intense reactions for alkaline phosphatase in fall testes (figs. 5 and 6). ×140.

to be located in either spermatogonia or spermatocytes, they are probably situated in the cytoplasm of Sertoli cells. The lipid droplets are not birefringent, but the sperm tails and cell membranes are, a birefringence which is not abolished by extraction with acetone. In the testis of a November Virginia deer examined in ultraviolet light,

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PLATE 3

Explanation of Figures

10. A group of interstitial cells from the testis of a Virginia deer (Oct. 19). Formalin-fixed, frozen section stained with sudan black B for 7 minutes. Compare with figure 1. ×10 ocular; =40 objective.

11. Another section from the same testis stained with Schiff's reagent, showing the depth and intensity of the plasmal reaction. Compare with figure 4. Observe that the plasmal reaction coincides in its pattern with that of the lipid in the previous section. ×10 ocular; ×40 objective.

12. A group of interstitial cells from a June testis (Virginia deer). Formalin-fixed, frozen section stained with sudan black B for 7 minutes. Compare with figure 10, observing the greatly diminished quantity of stainable lipid in the June testis. The plasmal reaction of the June testis (not illustrated) was similarly diminished. ×10 ocular; ×90 objective.

13. The epithelium of the distal portion of the ductus epididymidis showing the distribution of alkaline phosphatase. Virginia deer on Oct. 19. The borders of the columnar cells and the stereocilia react strongly as does also the cytoplasm of the small basal cells. Fixation in chilled 80% alcohol. Section incubated with fructose diphosphate at pH 9.4 for 3 hours. Counterstained with paracarmine. Compare with figures 23 and 27. ×10 ocular; ×90 objective.

14. The epithelium of the proximal portion of the ductus epididymidis of the same animal showing the distribution of acid phosphatase. The nuclei of 2 migrating lymphocytes are visible in the epithelium. The terminal bars at the surface of the epithelial cells react intensely. The stereocilia are faintly stained, and to the right deeply stained spermatozoa are visible. Fixation in chilled acetone. Section incubated in sodium glycerophosphate at pH 4.5 for 24 hours. Counterstained with paracarmine. Compare with figure 30. ×10 ocular; ×40 objective.

15. Spermatids from a seminiferous tubule of a Japanese deer killed on November 13. Fixed in Rossman's fluid and stained by the periodic acid-Schiff method. The acrosomes and portions of the acroblast applied to the nucleus react positively—staining not prevented by saliva and hence not attributable to glycogen. ×7 ocular; ×90 objective.

16. Spermatozoa (from ductus epididymidis) stained by the periodic acid-Schiff technique following fixation in Rossman's fluid. Virginia deer on Oct. 19. ×10 ocular; ×90 objective.

17. Spermatozoa from the same deer, fixed in Zenker's fluid and stained by the Feulgen method revealing the amount and distribution of desoxyribonucleic acid. ×10 ocular; ×90 objective.

18. Spermatozoa of a Japanese deer (Nov. 13) fixed in Zenker's fluid and stained with eosin and methylene blue. ×15 ocular; × 90 objective.

19. Spermatozoa of a Virginia deer (Oct. 19) fixed in Rossman's fluid and stained with Masson's triacid mixture. ×15 ocular; ×90 objective.

20. Spermatozoa of the same deer fixed in 4% basic lead acetate and stained in a ½% aqueous solution of toluidin blue, illustrating the absence of any metachromatic staining. ×7 ocular; ×90 objective.

21. Spermatozoa in the ductus epididymidis of the same deer showing the alkaline phosphatase reaction after incubating a section with fructose diphosphate at pH 9.4 for 6 hours. Fixation in chilled 80% alcohol. ×15 ocular; ×90 objective.

22. Spermatozoa in the ductus epididymidis of a Japanese deer in November stained for acid phosphatase. Section incubated with sodium glycerophosphate at pH 4.7 for 48 hours. Fixed in chilled acetone. ×15 ocular; ×90 objective.

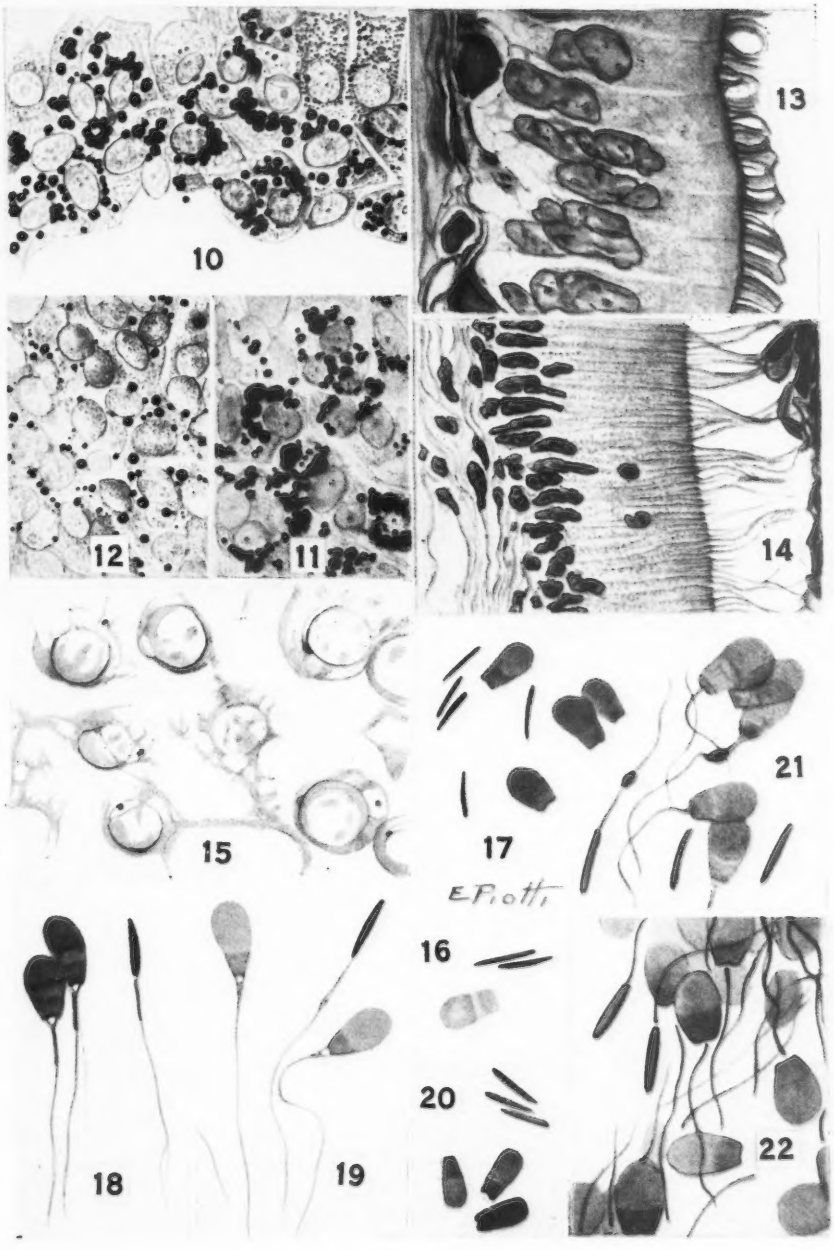

PLATE 3

a number of reddish-yellow granules and droplets were observed in the basal portion of the tubules. The yellow color, interpreted as being due to lipochrome, seemed to be associated with the coarser sudanophil droplets observed in the preparations stained with sudan dyes. A few similar reddish-yellow droplets were present in the interstitial tissue. The epithelial cells of the seminiferous tubules do not give a specific plasmal reaction (fig. 4), and the spermatozoa are quite unstained. In the inactive epithelium of the June tubules the lipid droplets are fewer in number and smaller in size.

In fall testes cytoplasmic basophilia is evident in the cells of the seminiferous tubules in eosin-methylene blue preparations. Basophilia is slight in the spermatogonia and spermatocytes, but increases in amount in the cytoplasm of the spermatids. In the globoid cytoplasmic residues which become detached from the spermatids it forms a delicate blue stippling on a faint lavender background. In June testes cytoplasmic basophilia is minimal.

In the tubules of fall testes the periodic acid-Schiff method brings out a variety of stained structures (fig. 3). The basement membranes are intensely stained, and within occasional tubules vivid red droplets are present in some of the Sertoli cells. Toward the centers of the tubules, staining of spermatids and of spermatozoa is observed (fig. 3, 15 and 16). In the spermatids red material is visible in hemispherical contact with the nuclear membrane in addition to a deeply stained red body which is either close to or in contact with the nucleus (fig. 15). The former is interpreted as being part of the acrosome and the latter as the acroblast. The heads of the spermatozoa appear in profile as red spindles and in full view as paler spatulate objects (figs. 3 and 16). In addition, pale stained material is seen in the tubular lumen, more especially in those segments of the tubules in which spermatozoa are differentiating. This stained substance is interpreted as being residual cytoplasm released from spermatids in the course of their cytomorphosis into spermatozoa.

Except for the dark red droplets present in some of the Sertoli cells, none of the elements mentioned above is affected by the use of saliva. Thus, only the former are regarded as being glycogen. Of the various periodic acid-Schiff positive elements described above, the Bauer-Feulgen technique, stains only the droplets of glycogen.

In the inactive June testes, some of the shrunken seminiferous tubules contain flocculent material in the tubular lumens, which is intensely colored by both the periodic acid-Schiff and Bauer-Feulgen methods. The solubility of this substance in saliva indicates that it is glycogen. The residual spermatogenic cells do not appear to be specifically stained.

During the fall the seminiferous tubules exhibit most intense reactions for alkaline phosphatase (figs. 5 and 6). Besides the basement membranes, the epithelial elements of the tubules stain deeply. The

reaction involves the cytoplasm of spermatogonia, spermatocytes and spermatids, being relatively faint in the spermatogonia and reaching extreme intensity in the spermatids. The nuclei also contain alkaline phosphatase, although the dark nuclear staining apparent in figures 5, 6 and 9 is attributable partly to the counterstain. The spermatozoa are also stained. In the sperm heads, seen in profile, it appears as though the enzyme were located in a cytoplasmic membrane enclosing the nucleus, because not infrequently a central nuclear area stained by paracarmine can be faintly recognized surrounded by a brown sheath. It seems also as though the reaction were present in the cytoplasm of the Sertoli cells between the germinal cells, especially in the regions where the spermatids and spermatozoa are attached. Finally, a reaction is present in the cytoplasmic material which is liberated into the lumen of the tubules during the cytomorphosis of the spermatids into spermatozoa. In the shrunken tubules of June testes the reaction for alkaline phosphatase is considerably reduced (fig. 9). The enzyme is present for the most part free in the lumens of the tubules and in the cytoplasm of the surrounding epithelial cells. There is none in the basement membranes. In several specimens the centers of the atrophic tubules contained somewhat more than figure 9 indicates.

Acid phosphatase is present in the tubules of fall testes, occurring both in the nuclei and cytoplasm of the cells (fig. 7). None is encountered in the basement membranes. Some is present in the cytoplasm of the spermatogonia, slightly more is visible in the spermatocytes and still more is present in the spermatids and spermatozoa. The rather intense staining of the nuclei is associated mainly with the chromosomes. There appears to be some also in the seemingly fibrillar, distal cytoplasm of the Sertoli cells, as well as a widespread, faint, punctage reaction in the lumens of the tubules. In June testes no acid phosphatase has been encountered (fig. 8).

EPIDIDYMIS

Observations on the epididymis have been confined principally to

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Explanation of Figures

23. Ductus epididymidis of a Japanese deer in November stained for alkaline phosphatase. Section incubated in sodium glycerophosphate at pH 9.4 for 6 hours. Fixation in chilled 80% alcohol. Figure 13 shows a detailed view of such a preparation. ×140.

24. The same from a deer killed in June, illustrating the complete absence of alkaline phosphatase. ×140.

25. Ductus epididymidis of a Japanese deer in November stained for acid phosphatase. Section incubated in sodium glycerophosphate at pH 4.7 for 48 hours. Fixation in chilled acetone. ×140.

26. The same from a deer killed in June, illustrating the complete absence of acid phosphatase. ×140.

27. Ductus epididymidis of a Virginia deer in October stained for alkaline phosphatase. Section incubated in fructose diphosphate at pH 9.4 for 6 hours. Fixation in chilled 80% alcohol. ×190.

28. A ductus efferens from the same section as the preceding, illustrating the distribution of alkaline phosphatase. ×190.

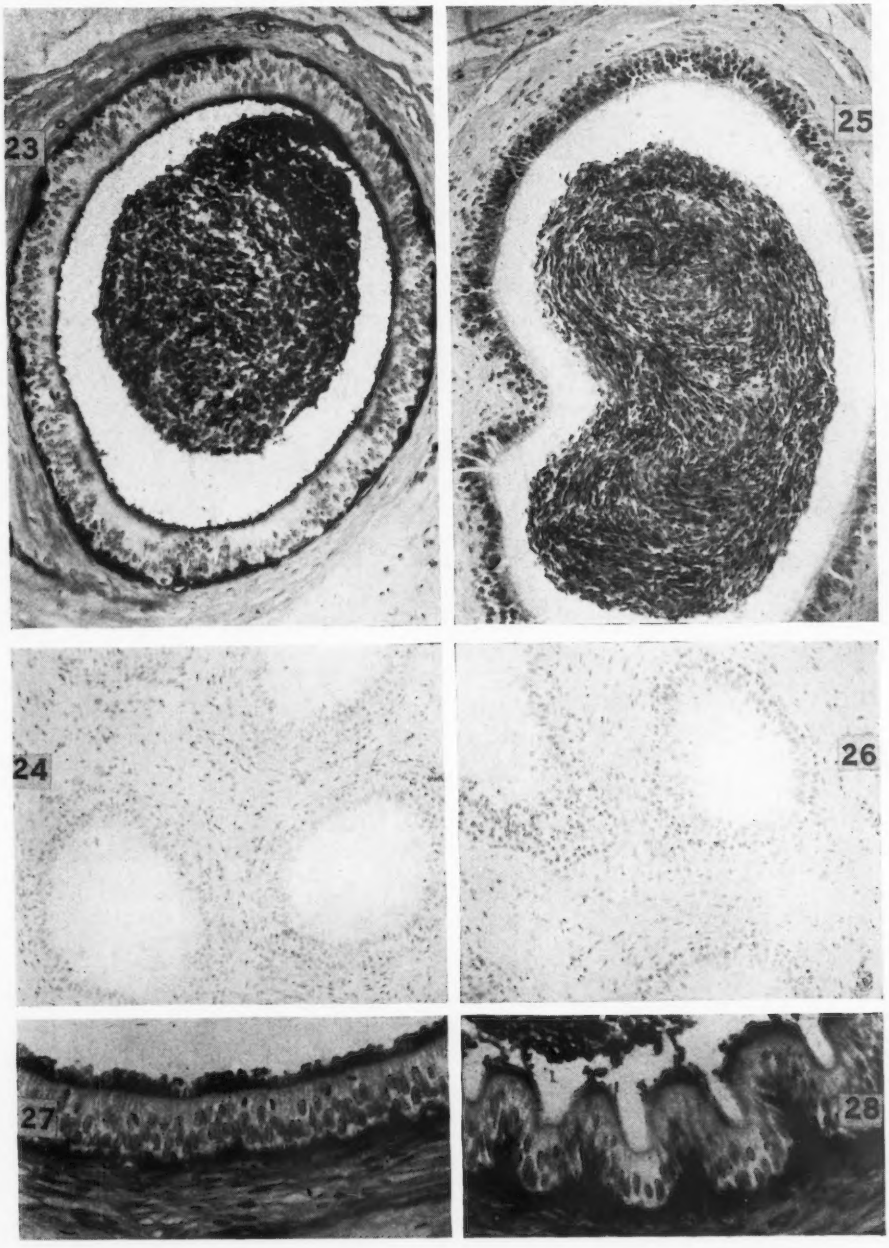

PLATE 4

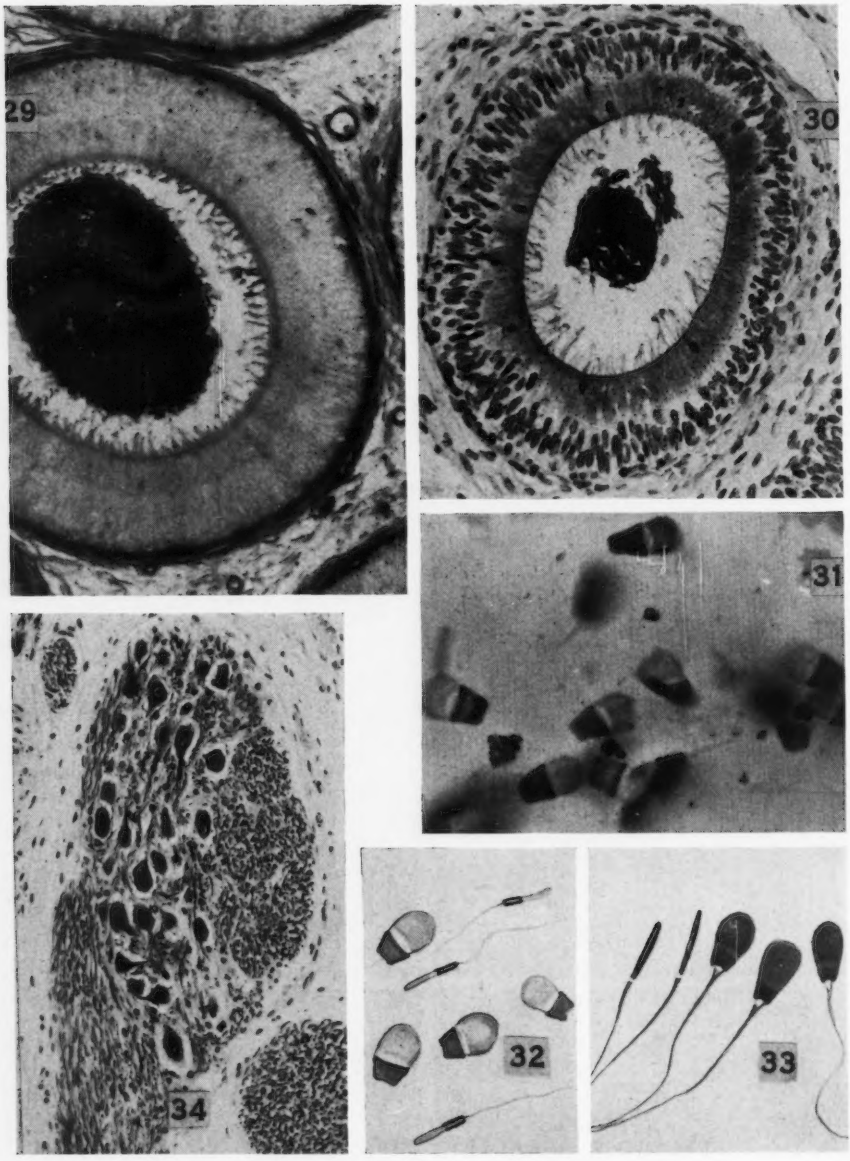

PLATE 5

the ductus epididymidis, but since a few of the sections have included some of the efferent ducts, these will be occasionally mentioned.

Ductus epididymidis. This duct appears to vary some in different parts of its length. We have not investigated these differences except to note that the epithelium of the proximal highly convoluted portion of the duct possesses taller cells bearing longer and more delicate stereocilia (figs. 14, 29 and 30) than the distal part where the cells and cilia are lower (figs. 13, 23, 25 and 27). In regard to histochemical features no differences have been noted in these 2 regions.

The epithelium of the ductus epididymides of the several deer examined contains relatively little stainable lipid. With sudan black B a few stained droplets are encountered in the small basal cells, but there are none in the columnar cells. No noticeable seasonal differences in regard to lipid have been observed.

In eosin methylene blue preparations after fixation in Zenker's fluid, the outer halves of the columnar cells including the border of stereocilia are strongly eosinophilic. In the deeper halves of the cells in the nuclear region a faint bluish lavender stippling is visible, indicating the presence there of some degree of cytoplasmic basophilia. The cytoplasm of the small basal cells is distinctly acidophilic.

In preparations fixed in basic lead acetate and stained with toluidin blue no metachromasia is visible in the various elements of the epididymis, excepting numerous mast cells in the stroma, which are strongly metachromatic.

The nuclei of the columnar epithelial cells are elongated and vesicular, whereas those of the basal cells are slightly shrunken and possess a relatively dark nuclear membrane. In addition to these 2 types,

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Explanation of Figures

29. Ductus epididymidis of a Virginia deer (Oct. 19). Fixed in Rossman's fluid and stained by the periodic acid-Schiff method. The basement membrane, the walls of the blood vessels and the mass of spermatozoa within the duct react strongly, but none of these reactions is prevented by exposure of the sections to saliva. Neither the epithelial cells nor the stereocilia are specifically stained. ×240.

30. Ductus epididymidis of a Japanese deer (Nov. 13) showing acid phosphatase in the nuclei, in the distal portions of the epithelial cells, in the stereocilia, and in the mass of spermatozoa within the lumen. The darkly stained nuclei of migrating lymphocytes are scattered throughout the epithelium. Fixed in chilled acetone. Section incubated in sodium glycerophosphate at pH 4.7 for 48 hours. Figure 14 shows a detailed view of this preparation. ×240.

31. Spermatozoa in the ductus epididymidis of a Virginia deer in October, showing their staining by Bodian's protargol method. Fixation in Rossman's fluid. ×1620.

32. Drawing of the same. Rossman's fixative and Bodian's protargol stain. ×15 ocular; ×90 objective.

33. Spermatozoa in ductus epididymidis stained with Heidenhain's iron-hematoxylin after fixation in Rossman's fluid. ×15 ocular; ×90 objective.

34. Sympathetic ganglion in capsule of seminal vesicle stained for acid phosphatase. Tissue fixed in chilled acetone and section incubated with sodium glycerophosphate at pH 4.7 for 48 hours. Counterstained with paracarmine. Observe the intense staining of the nerve cells and of the nerve fibers. ×160.

small, intensely chromatic nuclei are encountered scattered irregularly throughout the epithelium and present also in the tunica propria. With methylene blue or with Feulgen's stain these have the characteristics of the nuclei of lymphocytes. Some of them are surrounded by a clear halo of faintly stained cytoplasm. These cells are interpreted as being lymphocytes in process of migrating through the wall of the ductus epididymidis. Their nuclei contain acid phosphatase as illustrated in figures 14 and 30.

The long stereocilia, present on the cells in the proximal part of the epididymal duct, form bundles protruding into the lumen. The cilia do not possess basal bodies, but the surfaces of the columnar cells are equipped with terminal bars which produce a pattern of dots and lines indicating the cell boundaries. The terminal bars are readily seen after Zenker fixation and Masson's stain. In acid phosphatase preparations (figs. 14 and 30) the enzyme appears to be concentrated in or on the substance of the bars (figs. 14 and 30). Its appearance might be construed as related to basal bodies but analysis of the situation implicates terminal bars.

Seasonal differences in the cytology of the ductus epididymidis are slight, as revealed by eosin and methylene blue or Masson's stain. The major difference is the absence of spermatozoa in the epididymis of early June. Although the duct seems to be much narrower than in the active period, the epithelium is quite tall and is provided with normal appearing cilia. The nuclei of the basal cells seem to be more conspicuous than in the fall.

Stained by the Bauer-Feulgen method the ductus epididymidis is negative. After staining by the periodic acid-Schiff technique its appearance is illustrated in figure 29. The epithelial cells including the stereocilia are unstained. However, the basement membrane enclosing the duct and the mass of spermatozoa contained within the lumen are deeply colored. Since none of this staining is prevented by exposure to saliva, it is not due to glycogen.

In preparations of the epididymis from the fall months an intense alkaline phosphatase reaction is encountered in the stereocilia and in the cytoplasm of the small basal cells which form a discontinuous layer on the inner surface of the basement membrane (figs. 13, 23 and 27). The basement membrane and the well-defined muscle layer surrounding the duct react moderately (fig. 27). The nuclei do not react. The mass of spermatozoa contained in the duct lumen is very intensely colored (fig. 23). Alkaline phosphatase is also abundant in the efferent ducts (fig. 28). The reaction is intense in the basement membrane and in the underlying layer of muscle. On the free surface of the epithelium it occurs particularly on the crests of the scalloped folds. In June, when spermatogenesis is in abeyance, alkaline phosphatase is not encountered in the epididymis.

In the fall, acid phosphatase is also readily demonstrable (figs. 14, 25 and 30). Its distribution is different from that of alkaline phos-

phatase. It occurs principally in the cell nuclei of the epididymal duct and does not bring out either the basement membrane or the muscle layer. It occurs also in the distal halves of the epididymal epithelium increasing toward the surface and outlining the terminal bars with great sharpness (figs. 14 and 30). The stereocilia are delicately stained. Similarly to alkaline phosphatase, it is present in considerable concentration in the mass of spermatozoa in the lumen of the ductus epididymidis (figs. 25 and 30).

In June epididymides, on the contrary, it is practically absent, giving only a faint tinge to the cell nuclei (fig. 26).

SPERMATOZOA

The methods used in the present study offer an opportunity to observe certain cytochemical properties of spermatozoa. Because the observations are varied and numerous, they are assembled here under a separate heading. Insofar as we are aware, spermatozoa have not been hitherto examined in reference to their staining by sudan black B, the periodic acid-Schiff method or Bodian's protargol method. The spermatozoa were available for examination in fixed sections of both testis and epididymis.

Sudan black B. With this dye the sperm heads appear to be faintly stained, indicating the presence of a lipid component (figs. 1 and 2) which, it seems reasonable to believe, may be contained in the sheath surrounding the head.

Schiff's plasmal reaction. By this method the spermatozoa remain unstained. Since this reagent, as we have used it, stains lipids of the interstitial tissue very distinctly (fig. 11), there is no apparent reason to doubt the validity of the negative finding for the spermatozoa. This qualifying statement is added because Marza ('31) has described a diffuse staining of spermatozoa in various mammals by the "plasmal or aldehyde reaction of Feulgen," a finding which we are unable to confirm.

Glycogen. Glycogen is not demonstrable in the spermatozoa of deer by either the Bauer-Feulgen or periodic acid-Schiff reactions. With the use of iodine, applied in 2 different ways, Marza ('31) claims to have found small quantities of glycogen in the middle piece of the spermatozoa of various mammals.

Periodic acid-Schiff method. This method colors the sperm heads quite distinctly (figs. 3 and 16), but does not stain the middle pieces or tails. The staining of the head of the spermatozoon is quite probably related to the staining of the acroblast and acrosome of the spermatids of the preceding stages (fig. 15). Since this staining of the germinal cells is not influenced by the use of saliva, it cannot be attributed to glycogen. Moreover, since none of these elements of the cells reacts metachromatically with toluidin blue, an acid mucopolysaccharide does not appear to be involved. A substance of unknown composition suggests itself, in which, upon oxidation with periodic acid, exposed

aldehyde radicles become stained by Schiff's reagent. Gersh ('48) has recently announced that the Golgi apparatus of certain cells contains a glycoprotein which gives a periodic acid-Schiff reaction. The material of the Golgi apparatus is known to become incorporated into the acroblasts and acrosomes (cf. Metz, '32), and it may be for that reason that these structures give a positive reaction. Since during subsequent cytomorphosis of the spermatids part of the material of the acroblast is cast off while other parts become associated with the sperm head, account would also be taken of the Schiff positive reactions of these 2 derivatives.

Basophilia. The condensed chromatin of the heads of the spermatozoa stains intensely with basic dyes (figs. 16 and 18) and by the Feulgen method (fig. 17). Basophilia is confined exclusively to the head of the spermatozoon (fig. 20) and coincides exactly, it appears, with the nuclear area of condensed chromatin which gives the specific Feulgen reaction for desoxyribonucleic acid. Accordingly, cytoplasmic basophilia attributable to ribonucleic acid appears to be lacking. Nor is there any metachromasia visible after fixation in basic lead acetate and staining with toluidin blue. Stained with Masson's mixture of acid dyes, the nucleus is faintly colored with orange G, and the middle piece and tail with light green (fig. 19). The middle piece and tail have a strong affinity for acid dyes, as is illustrated further by specimens stained with a mixture of eosin and methylene blue (fig. 18).

Phosphatases. The masses of spermatozoa within the lumen of the epididymis exhibit very intense reactions for both acid and alkaline phosphatases (figs. 23, 25 and 30). Examined under higher magnification the individual spermatozoa are much paler, but reveal nevertheless a distinct complement of both acid and alkaline phosphatases (figs. 21 and 22). The 2 enzymes appear to be present throughout the length of the spermatozoon but they differ in character in some slight respects. When prepared for alkaline phosphatase, the anterior half of the spermatozoon heads is stained more darkly than the posterior portion, whereas with acid phosphatase the reverse is true. Moreover, in the acid phosphatase preparations there seems to be a gap in the region of the neck piece where enzyme is lacking, whereas with alkaline phosphatase (fig. 22) the necks are stained. It is difficult to decide whether these enzymes are localized principally in the interior of the spermatozoa or mainly on their surfaces. Since alkaline phosphatase appears to be associated with the surface of the cells of both ductus epididymidis and ductuli efferentes (figs. 27 and 28), it is possible that the phosphatase associated with the spermatozoa may also be located on or in the surface layer. Indeed, it is sometimes evident, in spermatozoa seen in profile, that the paracarmine-stained nucleus of the head is ensheathed by a layer containing alkaline phosphatase. On the other hand, since acid phosphatase appears definitely to be a component of the nuclei of the antecedent spermatids, it seems probable that it is also located inside the nucleus of the spermatozoa.

Bodian's protargol method. By this technique the heads of the spermatozoa are very sharply stained (figs. 31 and 32). The acrosomic cap (galea capitis), which has the appearance of a thin, structureless membrane covering the anterior two-thirds of the head, is grey. Its posterior border forms a faint curved line across the head. The posterior nuclear cap covering the caudal third of the head is intensely impregnated with silver. Between the edges of the anterior and posterior caps a narrow, unstained, lens-shaped field is visible through which the eye detects the transparent interior of the head. Spermatozoa viewed in profile illustrate further the intensely black posterior cap, the faintly stained anterior cap and the unstained intervening zone (fig. 32) Posteriorly, the head terminates in a concave edge which coincides exactly with the border revealed by the Feulgen method or toluidin blue (cf. figs. 17 and 20). Occasionally the body and tail of a spermatozoon are just barely perceptible.

Heidenhain's iron hematoxylin. By this stain all parts of the spermatozoon excepting the neck are brought out (fig. 33). It reveals the topography of the sperm with much the same clarity as with eosin-methylene blue or Masson's triple stain (figs. 18 and 19). By all three of these methods a pyramidal-shaped, unstained area of the neck piece is visible just caudad to the concave posterior border of the head.

SEMINAL VESICLES

The accessory male sex glands of deer have not been identified or investigated in the present study, except for a pair of glandular organs situated near the base of the bladder. From their location and histology, we regard these structures as being seminal vesicles. Each consists of a firm, tortuous, glandular mass compactly ensheathed by connective tissue. Each varies from 2 cm. in length, in the sexually inactive period, up to 3 or 4 cm. in the fall, and has a diameter of about 1 cm.

The seminal vesicles undergo pronounced seasonal histological changes (Wislocki, '43). In June they present numerous small alveoli lined by relatively low cells. At this time the connective tissue stroma between the shrunken alveoli is relatively abundant. In the lumens of the acini, in this month, there is a small amount of seemingly inspissated secretion consisting principally of an amorphous, brownish-black substance. By October the gland is very much altered: the lumina are much enlarged and are filled with a copious globular and granular secretion which has replaced the scanty, brown secretion of the inactive period. The epithelial cells have become exceedingly tall, from an average of 18 to 20 μ in June having attained a height of about 40 μ. With the great enlargement of the acini the stroma has become stretched and thinned out and has assumed a more complex branching pattern. To the general histological features noted on a previous occasion (Wislocki, '43), the present study adds a few additional observations derived from histochemical methods.

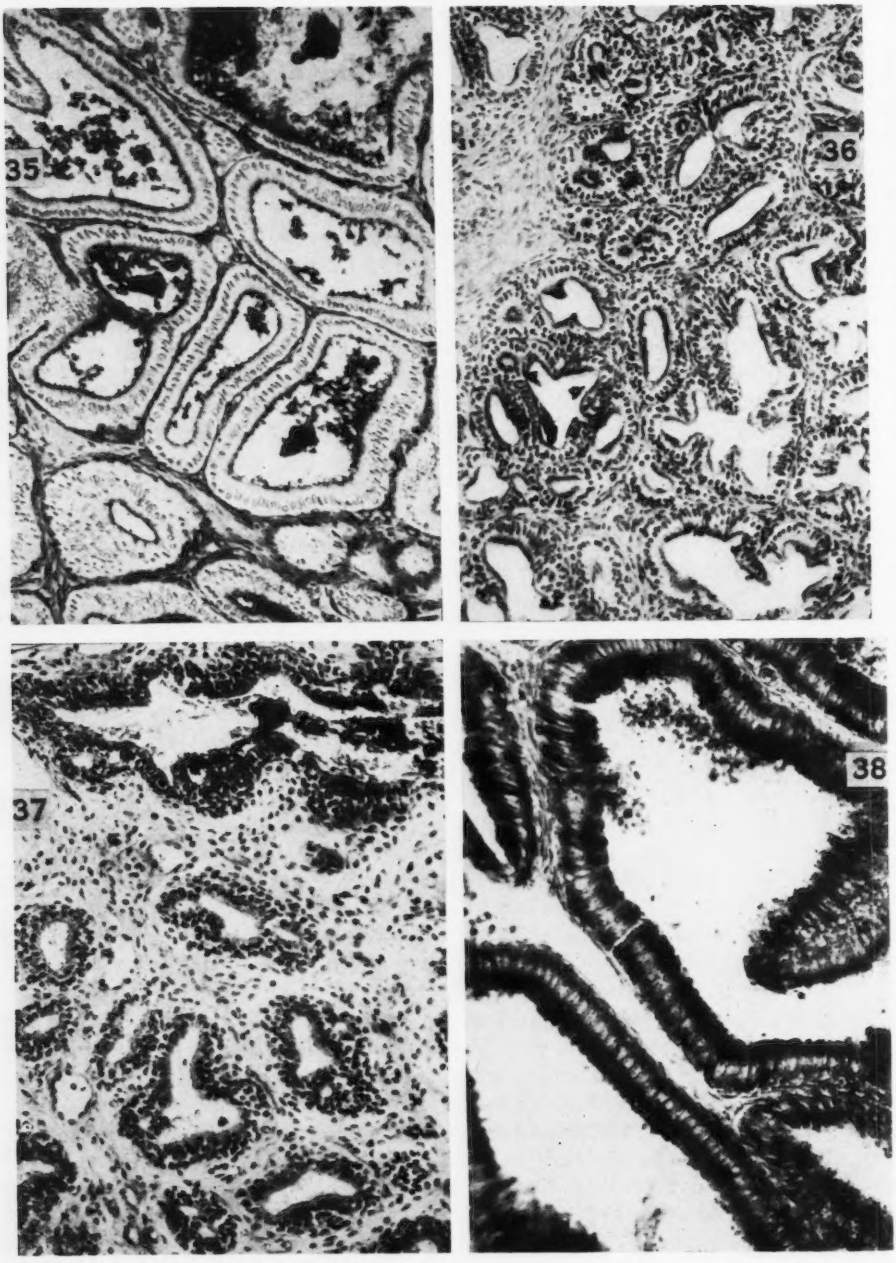

PLATE 6

Lipids are abundant in the epithelium of the fall seminal vesicles. In the Virginia deer the character of the lipid material is illustrated in figure 38. Minute sudanophilic particles are present in extreme amounts in the supranuclear and infranuclear regions of the cells. In a Japanese deer from the same period the character of the fat differs somewhat from that seen in the Virginia deer. Instead of a multitude of minute particles, the lipid occurs in the main in somewhat larger droplets located principally in the infranuclear region. In June testes sudanophil material is also present in the characteristic pattern for the species but in lesser quantities.

Cytoplasmic basophilia is not a feature of the epithelium of the seminal vesicles. Instead, the cytoplasm of the cells has a strong affinity for acid dyes.

After fixation in basic lead acetate and staining with toluidin blue, no metachromasia is visible, except for mast cells which are extremely numerous in the stroma. The granules of these cells are stained a deep purple.

There is some glycogen in the seminal vesicles, demonstrable by the Bauer-Feulgen and periodic acid-Schiff methods as checked by saliva controls. In specimens from the fall months it is present in the secretion in the lumens of some of the acini as well as also in the cytoplasm of some of the epithelial cells. In June specimens none is found free in the alveoli, but some is present in the cytoplasm of many of the epithelial cells.

Acid and alkaline phsophatases occur in the seminal vesicles. In the October gland alkaline phosphatase occurs abundantly at the distal margins of the epithelial cells, in the secretion filling the lumens and in the interacinar stroma (fig. 35). The apparent staining of the epithelial nuclei in the photograph is due to the use of paracarmine as a counterstain and not to phosphatase. In June much less alkaline

Explanation of Figures

35. Seminal vesicle of Virginia deer in October showing the reaction of alkaline phosphatase. Tissue fixed in chilled 80% alcohol and section incubated with sodium glycerophosphate at pH 9.4 for 6 hours. Counterstained with paracarmine. Observe the intense phosphatase reaction in the secretion, at the distal borders of the cells and in the stroma. The apparent staining of cell nuclei is due solely to the counterstain. ×140.

36. Seminal vesicles of Virginia deer in June for comparison with the preceding showing the reaction of alkaline phosphatase. Section prepared by the same technique as the preceding one. Observe in the June testes that the acini are smaller and the epithelium lower, and that there is little secretion in the lumens. The phosphatase reaction is limited to the distal ends of the epithelial cells. The nuclear reaction seen in this section is due solely to the counterstain. ×140.

37. Seminal vesicle of a Japanese deer in November stained for acid phosphatase. Tissue fixed in chilled acetone and section incubated in sodium glycerophosphate at pH 4.7 for 48 hours. Counterstained with paracarmine. The reaction of acid phosphatase, in contrast to that of alkaline phosphatase (fig. 35), is located principally in the nuclei. The nuclei are an intense brown or tan in the original preparation. ×140.

38. Seminal vesicle of Virginia deer in October. Frozen section of a formalin-fixed gland stained for 7 minutes with sudan black B. Finely divided sudanophil material is abundantly present in the epithelium in both supra- and infranuclear positions. ×220.

phosphatase is present (fig. 36). The shrunken gland lumens are for the most part empty, except for scattered masses of inspissated brownish secretion. A faint alkaline phosphatase reaction occurs in the distal cytoplasm of some of the epithelial cells and this becomes more intense along the free surfaces of the cells in some alveoli. In this photograph, too, the dark coloration of the nuclei is attributable to counterstaining with paracarmine. It is apparent that the October gland is richer in alkaline phosphatase than the June one.

Acid phosphatase is also present in the seminal vesicles, but, contrary to alkaline phosphatase, it is localized almost entirely in the nuclei of the epithelial cells (fig. 37). The darkly stained nuclei in the photograph are intensely brown or tan in the original preparation, in contradistinction to the nuclei of figures 35 and 36 which are red or pink in the original sections due to staining with paracarmine. Besides the acid phosphatase in the nuclei of the epithelium, there is a lesser amount of enzyme in the nuclei of the stroma. In the seminal vesicles of a June deer the acid phosphatase reaction is very much fainter.

In one of the fall specimens several sympathetic ganglia and a number of nerve bundles were encountered in the capsule of the seminal vesicles. The ganglion cells and the nerve fibers show intense reactions for acid phosphatase (fig. 34).

SUMMARY AND CONCLUSIONS

The testes, epididymides and seminal vesicles of deer undergo seasonal changes. In the present investigation these organs are compared in their active and inactive states with reference to lipids, steroid hormones, glycogen and acid and alkaline phosphatases.

The cells of the interstitial tissue in the fall contain lipid droplets which are sudanophil and birefringent, give a positive plasmal reaction, exhibit yellow fluorescence and are soluble in acetone. The combination of these reactions indicates that steroid hormones are formed in the interstitial tissue. In June testes these reactions are much less intense.

The interstitial cells contain traces of alkaline phosphatase and a little cytoplasmic basophilia, but no glycogen. The walls of the arterioles and the basement membranes surrounding the seminiferous tubules are rich in alkaline phosphatase but this disappears in the inactive period.

The cells of the seminiferous tubules of the active fall testes contain large amounts of both acid and alkaline phosphatases. Alkaline phosphatase is present in the cytoplasm of the germinal cells, being particularly intense in the spermatids. The nuclei are less deeply stained. Acid phosphatase occurs in both nuclei and cytoplasm of the germinal cells, the cytoplasmic reaction reaching maximum intensity in the spermatids. Both acid and alkaline phosphatases appear to be present in the cytoplasm of the Sertoli cells, and they occur also in the cytoplasm cast off from the spermatids into the lumen of the

tubules. In June these enzymes are much decreased.

Glycogen occurs in some of the Sertoli cells in active testes, while in inactive ones it is present in many of the tubular lumens. The acroblasts and acrosomes of the spermatids, as well as the heads of the spermatozoa, are stained red by the periodic acid-Schiff method, a reaction not prevented by the use of saliva and indicative of a glycoprotein.

The cytoplasm which is cast off by the spermatids contains lipid droplets, basophilic substance, alkaline and acid phosphatases, and a glycoprotein which reacts with the periodic acid-Schiff reagents.

The cells lining the ductus epididymidis contain both acid and alkaline phosphatases. The former is localized principally in the nucleus, distal cytoplasm and ciliated border of the columnar cells. The enzyme delineates the terminal bars at the surfaces of the cells. Alkaline phosphatase is localized in the ciliated border of the columnar cells and in the cytoplasm of the small basal cells. Lymphocytes, whose nuclei contain acid phosphatase, are seen migrating through the epithelium of the ductus epididymidis.

The spermatozoa exhibit various reactions. Their heads, besides being stained by the periodic acid-Schiff technique, are faintly stained by sudan black B. Both acid and alkaline phosphatases occur in the spermatozoa. The latter seems to be present in the cytoplasmic sheath covering the head. By Bodian's protargol method the heads are very sharply delineated: the anterior head cap stains grey and the posterior cap black, with a narrow intervening zone which is unstained.

Seasonal changes, in reference to size and secretory activity, are noticeable in the seminal vesicles. The seasonal distribution of acid and alkaline phosphatases, lipids and glycogen in these glands is briefly described.

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EARLY EFFECTS OF TESTOSTERONE PROPIONATE ON THE SEMINAL VESICLES OF CASTRATE RATS[1,2]

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THE LONG term effects of administered testosterone to castrate animals are well known. The synthesis of protein, the rise in acid phosphatase of the prostate gland (Huggins, et al. 1939), and the marked production of fructose in the seminal vesicles (Mann 1946a, 1946b) are well-recognized biochemical changes. Some histological studies have been carried out after the androgens have acted for short periods of time. Tislowitz (1939), using colchicine, demonstrated that profuse mitoses in the seminal vesicles of castrate mice occurred about 24 hours after the injection of testosterone. Burkhart (1939, 1942) has shown that testosterone propionate initiates hypertrophy of the cells in the prostate of the castrate rat 23 hours after injection, and cell division in 36 hours. The relation of these morphological changes to the biochemical phenomena, however, has not been explored. Experiments were therefore designed to determine at what time alterations in certain biochemical reactions appeared after injection of testosterone propionate.

METHODS

Male rats from Sprague-Dawley, Inc. were castrated when 30 to 33 days old. The preparation described by Hays and Mathieson (1945) was used, since they demonstrated that the seminal vesicles of these castrate rats exhibited the same increase in weight when the animals were injected with 1 mg. of testosterone propionate at any time from 7 to 350 days after castration. The treated rats were injected subcutaneously with a single dose of 1 mg. of testosterone propionate in 0.2 ml. of sesame oil. The seminal vesicles were weighed on a Roller-Smith microbalance immediately after their removal from the animal. The water content of the glands was determined by drying the tissues to a constant weight at 85°C. to 90°C., the barometric pressure usually being about 630 mm. mercury. The chloride content was determined by the micro-diffusion method described by Clark, Levitan, Gleason, and Greenberg (1942). The chloride which diffuesd into the potas-

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[2] From a thesis submitted by Guilford G. Rudolph to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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sium hydroxide was then determined by the iodometric method of Van Slyke and Hiller (1947). The sodium content of the glands was determined by the method of Leva (1940). This procedure was carried out in the cold room (temperature about +2°C.). The oxygen consumption was measured in the Warburg apparatus at 37°C. The tissues were kept in ice-cold buffer solution from the moment of weighing the glands. The glands were homogenized and the protein precipitated with trichloracetic acid. Aliquots of the protein-free filtrate were used for the fructose determinations. The fructose content of the seminal vesicles was determined by the method of Reinecke (1942).

RESULTS

Greene and Burrill (1940) noted an increase in the weight of the seminal vesicles 24 hours after injection. A maximum response to a dose of 1 mg. occurs 96 hours after injection (Hays and Mathieson, 1945). The data in Table 1 indicate that the increase in weight of the seminal vesicles of castrate rats 10 hours after the injection of testosterone propionate is significant as also are those 15 and 20 hours after injection. The calculation of an "overall t value" for the 5 groups of animals 10 hours after injection gives a value of 8.46, which is very highly significant.

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE ON THE WEIGHT OF THE SEMINAL VESICLES OF THE CASTRATE RAT

Hours after injection	No. of rats	Average weight of glands with S. E.	Average weight of glands from control rats with S. E.	Difference of average weights	t	P
		mg.	mg.	mg.		
5	5	6.70 ±0.30	6.33 ±0.25	0.37	0.91	0.4
10	30	8.67 ±0.21	7.33 ±0.15	1.30	4.84	<0.01
10	16	7.17 ±0.25	6.44 ±0.22	0.73	2.18	0.05
10	21	8.04 ±0.18	6.99 ±0.17	1.05	4.20	<0.01
10	24	7.66 ±0.17	6.65 ±0.17	1.01	4.31	<0.01
10	21	7.45 ±0.17	6.69 ±0.17	0.76	3.20	<0.01
15	5	7.66 ±0.35	6.33 ±0.25	1.33	3.18	0.02
20	36	9.53 ±0.22	6.63 ±0.17	2.90	10.53	<0.01

The data summarized in Table 2 show that the water content of the seminal vesicles is significantly increased 10 hours after the injection of the hormone. There is a small, insignificant decrease in chloride concentration. The water content 20 hours after injection is greater and is accompanied by a significantly decreased concentration of chloride. There is also a decreased sodium concentration 20 hours after the administration of testosterone propionate.

Table 3 gives the distribution of the water in the seminal vesicles of castrate rats before and after the administration of the androgen, based on the analytical data in Table 2. The increase in the "chloride space" at the 10-hour period was 14%. The "non-chloride space" increased 26% during the same period. At the 20-hour period the increases were 22% and 97%, respectively. The increases in "sodium

TABLE 2. EFFECT OF TESTOSTERONE PROPIONATE ON THE WATER CONTENT, CHLORIDE AND SODIUM OF THE SEMINAL VESICLES

	No. of groups of four rats each	Control	Ten hours after injection	Twenty hours after injection	t	P
		Average with S.E.	Average with S.E.	Average with S.E.		
% water	6	80.8 ± 0.15	82.1 ± 0.18		5.49	<0.01
% water	7	79.7 ± 0.64		82.0 ± 0.44	2.96	0.02
Chloride, meq./l. tissue water	6	84.9 ± 1.41	85.6 ± 1.09		0.74	0.5
	2	91.4 ± 3.10		80.9 ± 0.45	4.22	0.05
	3	95.1 ± 2.52		82.8 ± 1.26	4.31	0.02
Sodium meq./l. tissue water	2	135.5 ± 1.5		115.0 ± 3.2 (3 groups of rats)	5.35	0.02

space" and "non-sodium space" at the 20-hour period were 17% and 142%, respectively, With these calculations of "chloride space" and "sodium space" it is evident that the gland during the first 20 hours of growth incorporates most of the increased water into the cell contents.

With the increase in cell volume there is an accompanying increase in the organic constituents. In Table 3 it can be seen that the dry weights of the glands increased 6.4% during the first 10 hours and 22% during the first 20 hours. While there was a small increase in the amount of inorganic ions, these would not account for more than 10% of the change; the greater part of the gain must have been in organic compounds.

The data obtained from a number of experiments on the oxygen uptake of the seminal vesicles of castrate rats before and after treatment with testosterone propionate are shown in Table 4. When the oxygen consumption was measured with no added substrate the Q_{O_2} of the seminal vesicles from the animals treated with the hormone for ten hours was 5.37 and the Q_{O_2} of the glands of the untreated castrate controls was 4.21. The difference was statistically significant. The Q_{O_2} for the seminal vesicles 20 hours after the injection of testosterone propionate and for slices of seminal vesicles from normal mature rats did not differ significantly from that found 10 hours after injection. The

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON THE DISTRIBUTION OF WATER IN THE SEMINAL VESICLES

	Weight of Seminal Vesicles[1]	Dry weight	Water	"Chloride space"[2]	"Non-chloride space"	"Sodium space"[3]	"Non-sodium space"
	mg.	mg.	mg.	mg.	mg.		
Control	26.59	5.13	21.46	16.1	5.4		
Inj. 10 hours	30.63	5.46	25.17	18.4	6.8		
Control	27.61	5.16	22.45	17.6	4.8	18.9	3.6
Inj. 20 hours	37.17	6.30	30.87	21.4	9.5	22.2	8.7

[1] Total weight of the glands from four animals.
[2] Calculated from chloride values in Table 2, assuming all of the chloride is extracellular.
[3] Calculated from sodium values in Table 2, assuming all of the sodium is extracellular.

metabolic state of the gland is increased soon after the adminsitration of the hormone, and this level apparently persists as long as the hormone is present.

TABLE 4. EFFECT OF TESTOSTERONE ON THE Q_{O_2} OF THE SEMINAL VESICLES

No. of determinations	Substrate	Hours after injection	Q_{O_2}*	Q_{O_2}* of controls	Difference	t	P
2	glucose	5	3.0	3.2	−0.2		
23	none	10	5.37 ±0.23	4.21 ±0.23	1.16	3.52	<0.01
7	glucose	10	5.58 ±0.54	3.85 ±0.38	1.73	2.60	0.02
10	sodium succinate	10	6.79 ±0.18	5.51 ±0.26	1.28	3.78	<0.01
7	glucose +sodium succinate	10	6.59 ±0.46	5.65 ±0.85	0.94	0.97	0.4
4	glucose	20	5.24 ±0.18	3.01 ±0.28	2.23	6.76	<0.01
4	glucose	slices of gland from mature rat	5.66 ±0.45	3.00 ±0.46	2.66	4.07	0.02

* c. mm. O_2 per mg. dry weight per hour.

When glucose was added as substrate there was no appreciable change in the Q_{O_2} of either the treated or the untreated groups; the difference between the two groups was again significant.

When sodium succinate was added, however, the oxygen uptake of each group showed an increase of similar order; the Q_{O_2} of the seminal vesicles of the injected group remained significantly higher than that of the controls. When both glucose and sodium succinate were added the results were similar to those with succinate alone.

It was found that the addition of sodium fumarate did not increase the Q_{O_2} of either the treated or untreated glands. This unexpected result was followed by experiments on the inhibition of oxygen uptake by sodium malonate. The addition of sodium malonate caused a 43% decrease in the Q_{O_2} of the control glands, and a 37% decrease in the Q_{O_2} of the treated glands (Table 5). The addition of 0.01M sodium fumarate to the system inhibited by the malonate did not affect the oxygen uptake.

In two experiments on anaerobic glycolysis it was found that the amount of acid produced was similar for the seminal vesicles from the treated and the untreated animals. The $Q^{N_2}_{CO_2}$ for the glands from the animals injected 10 hours before sacrificing was 7.5 and the $Q^{N_2}_{CO_2}$ of the

TABLE 5. EFFECT OF SODIUM MALONATE ON THE Q_{O_2} OF THE TREATED AND UNTREATED SEMINAL VESICLES

Effect of the addition of sodium fumarate to the malonate-inhibited system

	Q_{O_2}	Q_{O_2} with malonate	Q_{O_2} with malonate +fumarate
Control	4.2	2.4	2.7
Injected	6.0	4.0	3.7
	6.3	3.8	3.5

untreated controls was 7.0. These results indicate that the hormone does not alter the anaerobic glycolysis of the seminal vesicles to any marked degree.

In Table 6 the data show that the level of fructose in the seminal vesicles of the untreated castrate rats was lower than the level found 10 or 20 hours after the injection of testosterone propionate. In order to ascertain whether or not the increased fructose preceded the increased oxygen uptake, determinations were made at 5 hours and 8 hours. It was found that approximately the same level existed at these times as in the untreated gland. The increased fructose level is, therefore, found at the same time that the increased oxygen consumption is obtained. This would indicate a possible relation between the two processes.

TABLE 6. EFFECT OF TESTOSTERONE PROPIONATE ON THE FRUCTOSE CONTENT OF THE SEMINAL VESICLES

	No. of determinations	Fructose mg./100 gm. tissue
Control	4	9.1
Injected 5 hours	2	7.3
Injected 8 hours	2	9.9
Injected 10 hours	3	15.2
Injected 20 hours	1	15.7

DISCUSSION

From the results of these experiments it appears that the primary effect of testosterone is on the intracellular metabolism of the seminal vesicles. A definite shift in the metabolic pattern, demonstrated by an increased oxygen consumption per unit weight of tissue, occurs as early as any measurable change in weight or shift in water and electrolytes. Once this has occurred, the new tissue which is formed also has this metabolic pattern since the oxygen consumption per unit weight of gland remains about the same even in the gland of the mature male.

The change in fructose concentration in the gland paralleled the change in oxygen consumption. In the uninjected animal and at 5 and 8 hours after injection the concentration was similar to the upper range of normal plasma concentrations. It then rose abruptly at 10 hours to a level which was markedly above those reported for plasma; this was maintained at 20 hours after injection. Similar values are given by Mann (1946b) for the mature rat prostate and seminal vesicle. Since the pattern of change of oxygen consumption and fructose concentration with time parallel each other it is probable that the two are associated phenomena.

The increase in intracellular water is probably the result of the accumulation of smaller, poorly diffusible molecules in the cell under the influence of the metabolic change. Fructose is one such molecule; it apparently does not passively diffuse from the seminal cells since the levels are markedly higher than the circulating fluids. This is par-

ticularly marked in the bull and ram where levels are 5 to 10 times the total reducing sugar level of the plasma. Even in man the levels in seminal plasma may be above 200 mg. per 100 cc. (Mann 1946b). Other molecules may also contribute to the osmotic change.

The effects of testosterone on the seminal vesicles contrast with those of another steroid, estradiol, on the uterus. The weight gain of the uterus after the injection of estrogen is not continuous. According to Astwood (1938), 6 hours after estrogen administration the weight of the uterus reached a first maximum followed by a decrease in weight between the sixth and twelfth hours, with a subsequent gain to the maximum response at 30 hours. The weight gain of the uterus at 6 hours was due almost entirely to an increase in water. Talbot, Lowry, and Astwood (1940) calculated from the electrolyte content of the uterus before injection, at 6 hours, and at 30 hours, that 80% of the fluid increase at 6 hours was extracellular, and that the fluid pattern of the unstimulated uterus again existed at 30 hours, the time of maximum weight gain. Hechter, Krohn and Harris (1941, 1942) reported a change in the permeability of the uterine capillaries soon after the injection of an estrogen.

At 6 to 12 hours after the injection of estrogen Kerly (1937) reported a slight increase in the anaerobic glycolysis of the uterus with a greater increase at 24 hours. The oxygen uptake of the uterus was significantly greater 36 hours after estrogenic stimulation (Kerly 1940). Carroll (1942) found a significant increase at 24 hours with a further increase at 45 hours. Apparently the first effect of estrogens is to change the fluid environment of the cell, after which the metabolic activity induced by these changes restores the normal distribution of fluid and electrolytes. This is in contrast to the androgen here studied, which appeared to act directly on the organic metabolism of the glandular cells; this, in turn, led to shifts in cells, water and electrolytes.

The first effect, histologically, of testosterone propionate on the seminal vesicles of the castrate rat that has been reported is hypertrophy (Burkhart 1942). An enlargement of the cells of the glands was noted 23 hours after the administration of the hormone, while increased mitotic activity could not be demonstrated until the 31 to 35-hour interval. Hays and Mathieson (1945) have shown that the maximum weight response to a single dose of 1 mg. of testosterone propionate occurred at 96 hours. The changes which we have observed are those, then, of the hypertrophic period.

While these studies did not involve the period of most rapid mitosis there is good reason to think that the multiplication of cells is a sequel of the primary metabolic shift, and does not involve marked new changes. As already mentioned, the oxygen consumption and fructose production of the mature gland do not differ markedly from those found 10 hours after injection. The increase in organic matter during the early period also indicates that synthesis is already accelerated.

The effects of testosterone on the metabolic pattern of the seminal

vesicles and prostate appear to be similar. Barron and Huggins (1944) found that the anaerobic glycolysis of the prostate of the castrate dog was the same as that of the prostate of the normal dog and of the gland of the castrate dog that had received testosterone propionate for 30 days. They concluded that testosterone regulates the oxidative phase of carbohydrate metabolism since the Q_{O_2} of the prostates of normal dogs and of injected castrate dogs was higher than the Q_{O_2} of the glands of the untreated castrate dogs. Similarly the change in the seminal vesicles appears oxidative, the anaerobic metabolism remaining relatively constant.

SUMMARY

Ten hours after the injection of testosterone propionate into castrate rats the Q_{O_2}, weight, intracellular water, and fructose content of the seminal vesicles were increased. The Q_{O_2} of the glands 20 hours after injection and the Q_{O_2} of normal mature glands were similar to the Q_{O_2} at 10 hours. The fructose content also rose between the fifth and tenth hours; thereafter this concentration was maintained. The metabolic effects seem primary. The continued growth of the seminal vesicles and the further increase of the intracellular volume were probably secondary effects of the hormone since the increased oxygen uptake could furnish the energy necessary for synthesis and the alteration of fluid distribution probably was the result of increased intracellular osmotic pressure.

The anaerobic metabolism of the seminal vesicles was unchanged after the injection of testosterone propionate. Succinate as substrate increased the Q_{O_2} while the addition of glucose or fumarate had no effect.

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NOTES AND COMMENTS

HISTOCHEMICAL CHANGES IN THE ADRENAL CORTEX OF THE RAT IN ALLOXAN DIABETES

BENNETT and Koneff ('46) have shown recently that the adrenal cortex enlarges and contains increased quantities of lipid in rats made diabetic by injections of alloxan. Various considerations led these investigators to conclude that the adrenal changes were caused by the diabetic state and could not be ascribed to the toxic effects of alloxan.

A number of histochemical reactions for lipid substances are now available. Among these are several procedures which offer presumptive evidence for the presence of ketosteroids, since positive reactions are obtained in all of the endocrine glands which produce steroid hormones (cf. Demspey and Wislocki, '46), and since changes in the intensity of these reactions accompany altered physiological activity of the adrenal cortex (Deane and Greep, '46). The increased lipid in the adrenal cortex observed by Bennett and Koneff might represent a change in the steroid content of the gland. Accordingly, it was decided to apply the group of histochemical reactions to the adrenal glands of rats made diabetic by alloxan in an attempt to determine whether or not the increased lipid reflects a similar change in steroids.

MATERIAL AND METHODS

Adult rats of the Long-Evans strain, weighing 130 to 150 grams, were used. In addition, one experiment was done on rats of the Sprague-Dawley strain. The diet consisted of Purina fox chow. All animals received intraperitoneal injections of alloxan (200 mg./kg.) on each of two successive days. Blood sugar determinations by the method of Folin and Svedberg ('30) were made at autopsy. The blood sugar values from all diabetic animals ranged between 275 and 620 mg. per cent.

Paralleling the experiments of Bennett and Koneff, one group of rats, consisting of diabetic animals and their uninjected controls, was killed 72 hours after the first injection of alloxan. A second group was killed 3 weeks later. At autopsy, the adrenal glands were weighed and fixed in 10% formalin. The glands were washed for several hours in running water and frozen sections were cut at 15 μ. One section was mounted unstained for study of fluorescence and birefringence and others were stained with sudan IV and with the Schiff reagents. Similar sections were extracted with acetone and subjected to the same procedures.

RESULTS

The adrenals of the diabetic rats were consistently larger than those from the corresponding controls. The increase in size was similar to that found by Bennett and Koneff. It was reflected histologically by a thickening of the zona fasciculata. The zona glomerulosa remained essentially unchanged.

At 72 hours, the zona fasciculata of the Long-Evans rats exhibited an

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increased sudanophilia throughout its entire extent. The fat-free transitional zone between the glomerulosa and fasciculata was wholly or partially obliterated. Increased numbers of finely divided birefringent particles were observed in the fasciculata. These deposits were especially heavy in the outer half of the layer, but were present in the cells bordering upon the reticularis as well, although in lesser concentrations. The Schiff reaction was also more intense and extended deeper into the cortex than did that observed in the adrenal glands of control animals. Two of the glands from this group exhibited an intensified and broadened zone of green fluorescence in the fasciculata, but the remaining two were indistinguishable from the normal control glands.

Three weeks after the injection of alloxan, similar but less marked changes were visible. Two of the 7 experimental animals exhibited an increased sudanophilia of the adrenal cortex. Increased numbers of birefringent particles were seen in 5 of these animals. The Schiff reaction was intensified in 6. Four of the 7 adrenals exhibited more intense and broader zones of fluorescence in the fasciculata than did the control glands.

An almost exactly opposite result was obtained in the group of rats of the Sprague-Dawley strain. This group, also killed 3 weeks after the injection of alloxan, exhibited marked reduction in the lipids of the fasciculata as judged by all of the reactions.

In general, the results show a good correspondence among the various histochemical tests as well as between the blood sugar level and the magnitude of change in the appearance of the adrenal. The sudanophilia, however, seems to be more variable than are the results from the other methods. Several examples exist in which little alteration in sudanophilia occurred in adrenals exhibiting marked changes in birefringence, fluorescence and in the Schiff reaction. This result is in keeping with other observations on the adrenal gland (Bennett, '40) and ovary (Dempsey and Bassett, '43) in which under various conditions the amount of lipid present does not parallel the amount of material exhibiting steroid reactions.

DISCUSSION

The data presented above indicate that the lipid, present in increased quantities in the adrenal glands of alloxan-diabetic rats, exhibits enhanced reactions for ketosteroids. The adrenal cortex is now known to elaborate many specific steroid substances, and the present methods provide no means of distinguishing which particular compounds are involved. However, one clue exists which indicates that the carbohydrate-regulating, rather than the salt-conserving, principle of the adrenal is the one affected. This clue comes from the work of Deane and Greep ('46) who have shown that experimental situations causing a modified output of the carbohydrate-regulating hormones are associated with histological and histochemical changes in the zona fasciculata. Conversely, alteration of the electrolyte balance causes changes confined to the glomerulosa (Deane, Shaw and Greep, '48). These experiments lead to the thought that the carbohydrate- and salt-regulating hormones of the rat's adrenal are separately produced in the fasciculata and glomerulosa, respectively. Since the changes described above were confined to the fasciculata, it seems likely that the carbohydrate-regulating hormones of the corticosterone type were involved.

The difference in lipid content of the adrenals from the two strains of rats is of interest. The stimulus ordinarily causing increased lipid in Long-Evans rats invariably led to lipid depletion in Sprague-Dawley animals. Deane, Shaw and Greep ('48) have discussed the effect of stress on the adrenal gland, and have suggested that during mild stress the lipid and ketosteroid content of the cells increases, whereas in severe stress the discharge of droplets exceeds their rate of formation and the cellular content is diminished. The thought comes to mind that Sprague-Dawley rats have less resistance to the diabetic stress than do animals of the Long-Evans strain. This thought is in accord with the well-known hardiness of the Long-Evans strain.

SUMMARY

In diabetes caused by injecting alloxan into rats, the zona fasciculata of the adrenal glands was enlarged. Animals of the Long-Evans strain exhibited an increase in sudanophilia and in reactions indicative of ketosteroids. In Sprague-Dawley rats, the lipid and ketosteroid reactions were decreased. These changes are interpreted as indicating a condition of stress in which there is an increased demand for the carbohydrate-regulating hormone of the adrenal cortex.

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A NOTE ON THE ESTROGENS IN THE BILE OF PREGNANT WOMEN[1]

CANTAROW et al. reported in 1943 that the estrogenic activity of liver bile ("C" fraction), obtained by duodenal intubation of women at full-term pregnancy, was about three times that of the blood. This finding was cited in support of a previously (1942) postulated theory of an enterohepatic circulation of estrogens. Following this clue, Pearlman et al. (1947) succeeded in isolating estrone in crystalline form from the bile of pregnant cows. The authors next turned their attention to the bile of pregnant women with the object of isolating and identifying the estrogens contained therein. Accordingly, over 20 liters of bile were assiduously collected by duodenal intubation of women who were in the last trimester of pregnancy.

Bioassay values for individual specimens of bile were found to be in line with those previously reported by Cantarow et al. (1943). Thus, for example,

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three specimens of "B" bile (equivalent to gall-bladder bile) assayed for 4, 5 and 26.5 mouse units per ml. respectively; the corresponding "C" fractions (equivalent to hepatic bile) assayed respectively for 2, 5 and 12.5 mouse units per ml. These specimens when pooled with the "A" fractions (1.2 liters in all) assayed for 4 mouse units per ml. of bile.[2] Another specimen (0.45 liters) when similarly pooled assayed for 3 mouse units per ml. of bile. These figures are appreciably lower than those (average 22 mouse units per ml.) reported for gall-bladder bile of pregnant cows (Pearlman et al., 1947), which is not surprising because bile obtained by duodenal intubation is considerably more dilute than gall-bladder bile.

Procedures for the extraction and partitioning of the biliary estrogens were those previously (Pearlman et al., 1947) employed in this laboratory. The first step (extraction of the bile with butanol) gave a poor recovery of estrogenic activity in this instance, however. Thus, for example, a butanol extract of 1.2 liters of bile assayed for only 500 mouse units whereas about 4,500 mouse units were expected on the basis of bioassay performed directly

TABLE 1. DISTRIBUTION OF ESTROGEN* IN THE BILE OF PREGNANT WOMEN

Volume of bile extracted (liters)	Batch number	Butanol extract (m.u.)	Free phenols (m.u.)	Conjugated phenols (after hydrolysis) (m.u.)
13.9	I	9,500	2,000	14,600
8.9	II		800	3,300
22.8	I+II		2,800	

* Aqueous solutions of the butanol extracts and oily solutions of all other fractions were injected subcutaneously into adult spayed mice; a vaginal smear technique (Allen and Doisy, 1923) was used for bioassay.

on the bile; aqueous solutions of the bile or butanol extracts were injected subcutaneously into adult spayed mice for estrogen assay (Allen and Doisy, 1923). Such poor recovery of estrogenic activity was not encountered in the past when working with dog (Pearlman et al., 1948) or cow (Pearlman et al., 1947) bile under similar conditions. It is possible that labile estrogens are present in human bile; Werthessen, Baker and Borci (1948) claim to have detected labile estrogens in human and rat blood.

Tables 1 and 2 summarize the data on the distribution of estrogen in the bile of pregnant women. Both free and conjugated forms of estrogen are present whereas in the bile of pregnant cows the estrogen is almost entirely in the free form. The presence of estriol in human bile is suggested by the finding that the strongly acidic phenols of hydrolyzed bile extracts show some degree of estrogenic activity. However, due to the comparatively low content of estrogenic material in human bile, positive identification of this material could not be achieved. No estrogenic activity could be detected in the acid fractions of unhydrolyzed or hydrolyzed human bile. This makes it appear unlikely that acids of the doisynolic type are present in any significant

[2] It is worth mentioning for the sake of comparison that no estrogenic activity (i.e., less than 1 mouse unit per ml.) could be detected in the bile of non-pregnant women or of men.

TABLE 2. FURTHER FRACTIONATION OF PHENOLS

Batch No. of bile	Nature of material	Weakly acidic phenols		Strongly acidic phenols (m.u.)
		ketonic (m.u.)	non-ketonic (m.u.)	
I	conjugated phenols (after hydrolysis)	9,600*		5,000
II	conjugated phenols (after hydrolysis)	2,200	<500	100
I+II	free phenols	800	330	<500

* Total weakly acidic phenols.

amount; Miescher (1944) had made the interesting suggestion that such acids might be normally present in the organism.

An attempt to isolate neutral steroid hormones from butanol extracts of human pregnancy bile (about 23 liters) was abortive due to the lack of adequate amounts of material. Products of this type such as pregnanediol-3(α), 20(β), pregnanol-3(α)-one-20 and etiocholanediol-3(α),17(β) were recently isolated in this laboratory from the bile of pregnant cows (Pearlman and Cerceo, 1948).

ACKNOWLEDGMENT

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NEW BOOK

BIOCHEMISTRY AND MORPHOGENESIS. By Joseph Needham. Published Cambridge: at The University Press; New York: The Macmillan Company; 1942.—XVI+787 pp. $12.50.

Various circumstances have delayed our reviewing of Needham's great book. This delay of more than 5 years may be deplored as a precedent apt to inaugurate an era of sluggish digestion of scientific literature. On the other hand, a book like Needham's "Biochemistry and Morphogenesis," with an incredible amount of data, involved problems, and controversial discussions crowded into less than 700 pages and with more than 5,000 references, is hardly meant to be read from cover to cover within a few months. It may not, in fact, be a bad idea to use books of this type for a considerable period before attempting to review them.[1]

Similar to his 1931 "Chemical Embryology" Needham's new book aims at a correlation of chemical and morphological processes which occur during the development of the embryo. In "Biochemistry and Morphogenesis" the analysis reaches deeper strata, and the emphasis is on work published after 1931.

The arrangement of material is, in many respects, excellent. Some headings of chapters and subchapters are masterpieces of terse and yet tangible wording. On the other hand, Needham's attempt to divide the whole material into 3 major parts, entitled the Morphogenetic Substratum, The Morphogenetic Stimuli, and The Morphogenetic Mechanisms, is apt to produce a false impression of exactness. An arbitrary distribution of material among the 3 parts proved unavoidable. Respiration, including the energetics of differentiation (p. 574 ff.), is discussed in "Morphogenetic Mechanisms" while both nutrition and material combusted as source of energy (carbohydrate, protein, fat) are found under "Substratum" (p. 49). It seems to us that the metabolic properties of shell-enclosed eggs (pp. 48–63) and the standard mortality curves of chick embryos (pp. 63–66) belong to the "Mechanisms" rather than to the "Substratum," while the differential response to thyroxin of the forelimb bud and the adjacent gill-tissue in tadpoles (p.448) would have found a more adequate place with the "Substratum" than with the "Stimuli." In other cases, the emphasis is, probably, a matter of personal preference.

Part 1 ("Substratum") contains a survey of recent data on the chemical constitution of eggs and of their nutritional material, with frequent references to older data in "Chemical Embryology." Needham suggests that Parts 1 and 3 of "Biochemistry and Morphogenesis" should be read in conjunction with the earlier book.

"The egg and its environment" is a representative chapter of Part 1. "Cleidoic" (i.e. closed) eggs, like those of birds, are completely endowed with

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[1] I am grateful to my colleague Dr. S. B. Davis for comments and criticisms during the progress of this review, and I wish to acknowledge helpful personal communications by Prof. G. H. Bodine, Dr. E. W. Caspari, Prof. J. Holtfreter, and Prof. W. Landauer.

organic material, inorganic material and water; "non-cleidoic" eggs may either, from the beginning, contain all organic material, but receive inorganic material and water from the environment (e.g., sea-urchins and sand-crabs), or they may not be equipped with any nutritional material, but depend on the parental organism for continuous supply of everything (main representatives: mammals). These different conditions, and their relations to the aquatic, intermediate, or terrestrial environment are presented in a very clear way in Table 5. However, the table-heading "Evolution of the cleidoic egg" can hardly refer to *phylogenetic* evolution, since some species which are close together in ordinary taxonomy are found on opposite ends of the table, e.g., Scyllium (the dog fish) and Carcharias (a shark). Both are selachian fishes, but the first one is oviparous and the latter one viviparous, with striking differences in their biochemical development (Table 8).

Many readers will appreciate the data and pictures of the amazing tree frog whose tadpole stages are passed entirely within the egg. Treasures for endocrinologists are hidden at the bottom of page 43. Here Needham summarizes the work of the investigators at the Naples Biological Station who found the degree of dependence of selachian embryos on the mother to be correlated with a decrease in size of the maternal liver, the latter being controlled by hypophysis, thyroid and suprarenals.

Part 2 ("Stimuli") deals with inductors and organizers, with the relations of genes and organizers, and with gradients. The chapter which is entitled "A digression on plant galls" should convince any skeptic that chemical agents can indeed be responsible for the production of specific biological structures. However, up to the present, no one has been able to reproduce specific galls by any chemically defined substance, or even by a cell-free extract of an insect or its egg. Needham attributes this state of affairs to the relatively poor chemical technique which has been applied to the problem.

The subsequent 300 pages of Part 2 are centered around the "hierarchy of organizers in animal development." The 1st grade organizer, or "primary evocator," causes the appearance of the first axis of the embryo (gastrulation, formation of neural plate); examples of 2nd grade organizer effects are the induction of a lens by the eyecup, or of an ear-capsule by an ear vesicle; a 3rd degree organizer is at work in the induction of a tympanic membrane by the cartilage of the ear-capsule. The way in which the potentialities decrease with each subsequent grade of the "hierarchy" is illustrated by a model (Fig. 42, on p. 111) consisting of a series of cones on three different levels. A ball rolls from the top of the highest cone (=maximal potential energy) to its base, thus landing on the top of one of the next lower cones, from there rolls down to the base of this cone, etc. It is interesting to compare this austere model of equilibrium states with the picturesque railroad yard which was given in Needham's 1936 book "Order and Life."

About 100 of the 300 pages mentioned are devoted to amphibian morphogenesis, the classical subject of studies on induction and organizers. Unfortunately, the important fate-map of Vogt (Fig. 57) is reproduced on too small a scale, and the pictures illustrating movements (cell-streams) of vitally stained areas during amphibian gastrulation (Fig. 56) are still worse. It would have been worthwhile to render these basic pictures in colors. The black-white fate-maps of fishes (Figs. 179–181), although of secondary importance in this book, are printed in a more satisfactory way.

The germ-layers are disposed of with a few forceful arguments and the following conclusion: "Evidently the distinction between the germ-layers is a convenient morphological one with little or no physiological meaning" (p. 150).

In the chapters on biochemistry and chemistry of the primary organizer the inductive properties are discussed of living tissues, dead tissues, cell-free extracts, a number of impure substances and, finally, of well-defined substances like steroids.

Needham explains the disappointing "unspecificity" of the primary evocator (organizer) by the hypothesis that districts other than the dorsal lip of the blastopore may contain the "evocator substance" in an inactivated condition, and that the substance could be activated by unspecific mechanisms. A tentative list of such inactive complexes is given (pp. 206 and 207), and a way out of the dilemma is expected by closer attention to the question of the minimal effective dose. As a step in this direction a comparison is made between the effective doses of various evocating substances, of a cardiac drug, of several hormones and vitamins, and of two carcinogenics (Table 16, p. 184). Needham rightly emphasizes the difference between highly potent evocators such as 1, 2, 5, 6-dibenzanthrazene endo-α-β succinate, effective between 0.25 and 0.0025 mg./kg. wet weight, and weak evocators like nucleoproteins with an effective dose of 62500 mg./kg. wet weight. However, with respect to the other quite heterogeneous items of Table 16, a comparison is not only "a little difficult," as Needham puts it, but seems hardly possible to the reviewer.

A preliminary discussion of the metabolism of the gastrula anticipates the more detailed and final discussion in Part 3. A comparison between the evocator which induces the medullary plate (without cranial and caudal pole) and the auxin effect in plants is attempted in spite of the fact that the longitudinal growth of the cellulose walls in plant cells caused by auxin has no counterpart in the morphogenesis of animals.

In Needham's book abnormal organizer activities are considered responsible for three groups of formations which already in traditional human pathology have been treated as related phenomena, namely, twinning, teratomata, and malignant tumors. The greater part of these discussions (30 pages) are devoted to "organizers in cancer." Needham translates the old concepts of pathology into the new language. In his terminology the most important aspect of cancerous growth is that it is "an escape from the individuation field," whereby, in this case, individuation refers to any differentiation beyond the mere formation of a head- and tailless medullary plate. A large number of carcinogenic hydrocarbons and other substances are discussed with respect to their chemical and biological properties.[2] Diagram Fig. 138 shows 49 substances with their "overlapping domains" of different activities, for instance, 3, 4,-benzpyrene which is a primary evocator (neurogen), a carcinogen, and an estrogen. Unfortunately, colchicine is missing in the diagram, although present as No. 43 in the list of substances. Otherwise the diagram is excellent, and can be recommended for use in lectures.

[2] The much-discussed theory of the origin of malignant tumors from embryonic rests was abandoned not through "progress on biochemical lines" as Needham assumes (p. 240, footnote), but through recognition of the wide distribution of meristematic cells, i.e., tissue cells which, in the adult organism, maintain the ability of dividing.

Except for the determination of sex, little was known concerning the relations between genes and embryonic differentiation, when "Chemical Embryology" appeared in 1931. During the subsequent 10 years those relations have been investigated with so much success that they fill 90 pages of "Biochemistry and Morphogenesis." Genes which interfere with the action of the primary organizer are responsible for certain hereditary malformations of the heads in guinea pigs and chicks, and also for the short-tail malformation in mice, and the hereditary rumplessness in fowl. Genes interfering with the action of second-grade organizers are illustrated by examples like the genetic disturbances in the development of the eye or the limb-buds. A chapter "The origin of species specificity" contains examples of heteroplastic transplantations which document the fact of species specificity, but do not throw any light on its origin, as far as the reviewer can see.

The discussion on the role of the nucleus in embryonic development is based on hybridization, merogony, and parthenogenesis, with an excellent diagram (Fig. 205) and a very useful definition of terms (Table 24). A short survey of genes which control chemical processes is followed by a chapter on "nuclear inductors and nuclear metabolism." Here a series of splendid experiments with exchange of organs or fluids between different mutants of Drosophila is presented. Needham also gives a detailed description of studies on regeneration of Acetabularia, an alga which, in spite of its high degree of morphological differentiation and its size of 3 cms., contains only one nucleus. At the end of the discussion on genes and organizers the role of the sulfhydryl group and of glutathione is analyzed, mainly in the light of data obtained from large and small races of the same species, e.g., of rabbits and fowl. Needham points out that there are possible relations between glutathione and pituitary growth-hormone, but that the different rate of embryonic growth before the appearance of the pituitary remains unexplained, as the initial sizes of eggs are similar in small and large races.

The interrelations of genetical, endocrine and other factors are demonstrated by the frizzle condition in fowl, a hereditary malformation of feathers which disturbs the control of body temperature, and thus leads to changes of thyroid gland, food intake, intestinal length, blood picture, etc. Abnormal cartilage formation in rats, with its consequences, is another example showing the remarkable variety of phenomena which may be traced to one gene (Fig. 231).

The place of hormones in Needham's system will be of special interest to the readers of "Endocrinology." He classifies the products of endocrine glands as "the last of the three great inductor types," at least with reference to amphibial development. The two preceding types are the "true embryonic inductors" and the nuclear inductors derived from genes.

The imitation of gene-action by environmental factors ("phenocopies") is illustrated by several examples. The influence of genes and environmental temperature on the wing-pattern of the flour-moth (work of the Göttingen group) is discussed in detail, but the important relation between mitotic pattern and pigment production is not mentioned.

The last 50 pages of part 2 are devoted to organizer phenomena in insects, the metabolism in insect metamorphosis, and finally the determination in echinoderm development and metamorphosis. Needham reproduces some of the most important pictures from the studies of the Stockholm investigators

who demonstrated the double gradient system in the echinoderm egg. Improved recent experiments of Child and his collaborators are accepted as possible supports of the theory of *metabolic* gradients (pp. 496–497). Yet, Needham sees no reason why he should modify his negative attitude, as expressed in "Chemical Embryology," toward the experimental evidences of metabolic gradients, though he now praises Child for having introduced the concept (p. 605). An attempt to reconcile the organizer and the gradient theories concludes Part 2.

Part 3 ("Mechanisms") is introduced by an excellent chapter on the dissociability of the fundamental processes of ontogenesis showing how nuclear division can take place without cell division, cell division without nuclear division, differentiation of eggs without cleavage, etc. There are a few criticisms. On Table 28 "growth" is, without discussion, defined as "increase in spatial dimensions and in weight," although increase in volume, in dry weight, and in wet weight are dissociable processes, e.g., in the growth of tadpoles. In the list of fundamental processes the important migrations of cells and cell groups are missing. The dissociation of histogenesis from organogenesis is not made very clear (p. 514); malignant tumors could have been used as a striking illustration of this dissociation.

The unequal increase of different chemical components ("chemical heterauxesis") during embryonic development is illustrated in 12 charts and 4 tables, but there is little connection between these data and morphogenesis. The S-shaped growth curves of the whole body had been given much space in "Chemical Embryology," because of their resemblance to the curve of autocatalytic monomolecular reactions. In "Biochemistry and Morphogenesis" Needham devotes a few lines only to these curves stating rightly that their analysis "has been singularly lacking in fruitfulness or illumination." Nothing else could be expected, since mathematical resemblance alone does not indicate similar mechanisms; this was pointed out by G. Teissier (1928) and the reviewer (1933).[3]

The chapter on respiration deals with the early stages of eggs in echinoderms, and with various stages of embryonic development in arthropods, fishes, sauropsides and mammals. Particularly useful is a survey of recent pioneer work on the respiration of the grasshopper before, during and after the "diapause," which is a phase of morphogenetic dormancy. The discussion of carbohydrate metabolism covers, besides the data of other investigators, important contributions by Needham and his collaborators. The chapter on protein metabolism contains some pages on "The growth-promoting factor" of embryo juice as studied in tissue cultures. In the discussion on nuclein metabolism "complete synthesis of both phyto- and thymonucleic acid from non-cyclic precursors" is claimed for the embryos of birds and reptiles (p. 635). The meaning of "complete synthesis" is not clear to the reviewer; in any case the presence of nucleic acids in the unincubated egg is proved by the presence of its nucleus, though their amount may be too small for chemical detection. For lipin and sterol metabolism the reader is mostly referred to "Chemical Embryology," except for new work with tracer substances. Finally, the metabolism of various pigments is discussed.

The last 20 pages of Part 3 deal with "Protoplasmic Organization." Results of crystal-analysis of fibers are tentatively applied to the problems

[3] Arch. f. Entwicklungsmechanik, *130*, pp. 486–490.

of egg-cell structures. The characteristics of liquid crystals, or the paracrystalline state, are described in detail, and the biological significance of this state is emphasized. Among the proteins with highly anisometric molecules myosin (actomyosin, Szent-György) has moved into the center of interest. However, when "Biochemistry and Morphogenesis" appeared, only the first few studies on the relations between myosin and adenosinetriphosphatase had been published, including the 1941 paper by Needham, Shen et al. Under these circumstances some statements in "Biochemistry and Morphogenesis" seem almost prophetic, like that one on p. 670: "The probability is that molecular contractility plays a far greater part in development than embryologists have generally been willing to admit."

We have attempted to convey to the readers of "Endocrinology" an idea of the contents and the organization of Needham's book. The book is an expression of his creed that "structural alterations of the proteins are the basis of all morphology" (p. 605, footnote). Some bridges across "the secular gulf between morphology and chemistry" (p. XV) are, visibly, under construction on those pages which deal with "Protoplasmic Organization." In most of the other discussions the "bridges" still are in various stages of planning in spite of all the interesting correlations between biochemical and morphological phenomena of ontogeny. Needham's book gives an excellent account of the state of affairs.

Everyone will appreciate the fact that Needham tried to limit his gigantic task by excluding functional physiology as much as possible (see p. 643 footnote, on embryonic movements and reflexes). Yet, embryonic cells have to satisfy requirements for maintenance besides those for development; as G. L. Streeter used to put it, "embryonic tissues are open for business during alterations." This consideration would have been helpful in Needham's discussions of respiration and metabolism.

It is obvious that not all of the numerous data and techniques reported in "Biochemistry and Morphogenesis" could be handled with equal care. In connection with the regional respiration and glycolysis of gastrulae various micro-manometric techniques are compared in adequate detail; on the other hand, the absence of "the characteristic protein curve" in intact living sea-urchin eggs is stated (p. 656) without mentioning the limitations of spectrography in heterogeneous systems. Publications on tissue cultures are quoted without technical comments; a brief summary of these techniques is promised on p. 628, but not given. Unnecessary arguments are the result, as in the case of the lactogenic hormone. In the pigeon's crop in situ, the mitotic activity of the epithelium is increased by lactogenic hormone, but, according to Needham (p. 76), "this action could not be reproduced in vitro." The original papers show that the work in situ is valid, while the work in vitro is not: embryo juice had been added to all cultures in vitro so that the highest growth-rate possible was already obtained in the controls.

Sometimes, Needham's lack of interest in non-chemical procedures has led him to select poor papers when better ones were available. Abnormal turning of the 2–3 day-old chick embryo on its right side instead of its left occurs in 2 to 6% according to all authors who observed the embryo through an opening in the shell. The only paper quoted by Needham (p. 65) gives 0.163%, a pseudo-accurate figure based on mere candling of eggs on the 5th day (which is too late anyway).

The bibliography is an especially useful part of the book. Most of the references proved to be correct when checked. Only a few publications are misquoted. The paper by Crosman, quoted on p. 66, does not deal with the absorption of dead embryos in rodents, neither does the paper by Ancel et Vintemberger, quoted on p. 218, say anything about the possible effects of incident light on the plane of symmetry. On p. 629 Needham reports that the inhibition by "lipins" of the growth-promoting effect of embryo extract is reversed by heparin, while that is not so according to the original paper by E. Mayer.

The numerous graphs are very helpful. If their authors were stated in the legend, it would be clearer which graphs were made by Needham, modified by him, or reprinted unchanged. The "Glossary" gives a number of important definitions. However, "Gestalt" is antonymous to "the mere sum of parts," not to "surroundings." Could Driesch's old terms "prospective significance" and "prospective potency" not be replaced by routine potency, spare potency and total potency? The "Mitogenetic rays" are, to our deep satisfaction, on Needham's list of "Terms and concepts the use of which is not recommended."

The alphabetic index prepared by Margaret Miall is satisfactory with some exceptions. It is difficult to find the various places where axes and poles are mentioned. For large items like "Chick embryo and tissues" (30 different pages indexed, 2 of them wrong), and "Sea-urchin" (20 different pages indexed) some subindexing would have been useful.

Many interesting historical and linguistic remarks are scattered in the book. The reviewer takes it for granted that the Chinese sentences are correct, but would have preferred the absence of wrong German like the "erster Nährung" (instead of "erste Nahrung") on p.15. The verses which Needham ascribes to Goethe (p. 231) are from the "Lied von der Glocke" by Schiller. Names of American authors like Chaikoff and Romanoff should not have been altered into Chaikov and Romanov, irrespective of Needham's linguistic arguments (p. 692).

As stated before, it is evidently impossible to write great synthetic books like "Biochemistry and Morphogenesis" without a certain number of errors. Original papers are, as usual, to be consulted whenever a special point is of interest to the reader. In spite of all criticisms "Biochemistry and Morphogenesis" is an admirable and very useful book. We hope that it will help in attracting brilliant young workers to the promising field of experimental embryology.

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ASSOCIATION NOTICE

ANNOUNCEMENT OF THE 1949 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-First Annual Meeting of The Association for the Study of Internal Secretions will be held in the Chalfonte-Haddon Hall, Friday and Saturday, June 3, and 4, 1949, in Atlantic City, New Jersey.

We are informed by the hotel management that reservations will be difficult to secure on short notice; therefore, members are urged to make reservations at once with Chalfonte-Haddon Hall, giving time of arrival and length of stay in Atlantic City.

The scientific sessions will be held in the Viking Room, as formerly, and registration will be on the same floor. The annual dinner will be held in the Rutland Room, Friday, June 3rd. at 7 P.M., preceded by cocktails in the same room.

Those wishing to present papers, which will be limited to ten minutes, should send title and four copies of an abstract of not more than 200 words, to Doctor J. S. L. Browne, Royal Victoria Hospital, Montreal 2, Canada, not later than March 1, 1949. It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing in the program.

Nominations for the Squibb and Ciba Awards and the Ayerst, McKenna and Harrison Fellowship should be made on special application forms, which may be obtained from the Secretary-Treasurer, Doctor Henry H. Turner, 1200 North Walker, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1949.

POSTGRADUATE COURSE IN ENDOCRINOLOGY

A postgraduate course in Endocrinology, sponsored by the Association for the Study of Internal Secretions, will be held at the Skirvin Hotel in Oklahoma City, February 21–26, 1949.

The faculty will consist of outstanding clinical and research endocrinologists of the United States and Canada. The program will consist of clinics and demonstrations and will be a practical one of equal interest to those in general medicine and the specialists.

The fee will be $100 for the entire course and applications will be accepted in the order received. Applications should be directed to Henry H. Turner, M. D., Secretary-Treasurer, 1200 North Walker, Oklahoma City, Oklahoma.

ASSOCIATION AWARDS FOR 1949

THE E. R. SQUIBB AND SONS AWARD

The E. R. Squibb & Sons Award of $1,000.00 was established in 1939, and was given first in 1940 to Dr. George W. Corner; 1941—Dr. Philip E. Smith; 1942—Dr. Fred C. Koch; 1943—no award was given; 1944—Dr. E. A. Doisy; 1945—Dr. E. C. Kendall; 1946—Dr. Carl G. Hartman; 1947—Drs. Carl F. and Gerty T. Cori; 1948—Dr. Fuller Albright. A special committee of five members of the Association chooses an investigator or investigators in the United States or Canada for one of the best contributions to endocrinology.

THE CIBA AWARD

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than 35 years of age in the field of endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945—Dr. Jane A. Russell; 1946—Dr. Martin M. Hoffman; 1947—Dr. Choh Hao Li; 1948—Dr. Carl G. Heller. The work cited may be either in the field of preclinical or clinical endocrinology. The Award is for $1,200.00. If within 24 months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, the Award will be increased to $1,800.00.

THE AYERST, McKENNA & HARRISON FELLOWSHIP

The Ayerst, McKenna & Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin, and in 1948 to Dr. Ernest M. Brown, Jr. This Fellowship is designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. The Fellowship may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend for the Fellowship will vary in accordance with the qualifications of the appointee, but will not exceed $2,500.00 The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. The nominee must present evidence or scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals; submit a program of proposed study; indicate one or more institutions where the proposed program shall be carried out; submit statements of approval from the investigators with whom he proposes to conduct his research.

Each member has the privilege of making one nomination for each award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to endocrinology and by a bibliography of the nominee's most important publications, and reprints, if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City, Oklahoma, and returned to him not later than March 15, 1949.